Biphasic Effect of HMG-CoA Reductase Inhibitor, Pitavastatin, on Vascular Endothelial Cells and Angiogenesis

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Background HMG-CoA reductase inhibitors (statins) have pleiotropic effects beyond their cholesterol-lowering effect. However, consensus on the effect of statins on endothelial cells and angiogenesis has not yet been reached.

Methods and Results The effects of pitavastatin on the migration, proliferation and viability of human epidermal microvessel endothelial cells (HMVECs) were examined using scratch assay, chemotaxis chamber, bromodeoxyuridine incorporation, trypan blue dye exclusion test, and nuclear DNA staining. Pitavastatin enhanced the migration, proliferation and viability of HMVECs at a low concentration (0.01 μmol/L) but inhibited them at high concentration (1 μmol/L). The inhibitory effect on cell viability by high concentration of pitavastatin was recovered by geranylgeranyl pyrophosphate, but the effect on migration and proliferation was not. The cell activating effect of a low concentration of pitavastatin was reversed by both farnesyl pyrophosphate and geranylgeranyl pyrophosphate. A quail chorioallantoic membrane assay showed that high concentration (1 μmol/L) of pitavastatin reduced fibroblast growth factor-2-induced angiogenesis, whereas low concentration (0.3 μmol/L) tended to increase angiogenesis.

Conclusion Pitavastatin has a biphasic effect on HMVECs and on angiogenesis through at least 2 different pathways that include the mevalonate pathway. (Circ J 2005; 69: 1547–1555)

Key Words: Angiogenesis; Isoprenoids; Migration; Pitavastatin; Proliferation; Vascular endothelial cells

Statins reduce the serum cholesterol level by inhibiting 3-hydroxy-3-methyl coenzyme A (HMG-CoA) reductase. Mega-trials have demonstrated the safety and efficiency of long-term cholesterol-lowering therapy using low-dose statin and that intervention with statins prevent cardio- and cerebrovascular events in patients with hypercholesterolemia. It has been suggested that the preventive effect of statins is attributable to their cholesterol-lowering action, but there is research demonstrating that statin therapy also prevents cardiovascular events in patients with an average cholesterol level suggesting that statins prevent coronary vascular events not only through cholesterol-lowering but also through some other process. The mechanisms of these effects beyond the reduction of cholesterol, the so-called pleiotropic functions, have been gradually elucidated by a number of studies.

The pleiotropic functions of statins are involved in the regulation of cell adhesion molecules and the cell cycle inhibition of cell growth and/or induction of apoptosis in vascular smooth muscle cells inhibition of matrix metalloproteinase induction of endothelial nitric oxide synthase (eNOS) in vascular endothelial cells (ECs) and inhibition of vascular NAD(P)H oxidase among others. Moreover, we have reported that in experiments in dogs cerivastatin inhibited migration of ECs in vitro and intimal thickening of balloon-injured arteries in vivo.

With respect to angiogenesis, some researchers have show that simvastatin and cerivastatin promote collateral vessel formation in ischemic tissues of animal models. On the other hand, these same drugs reportedly inhibited the migration and viability of ECs, and interrupted angiogenesis. More recent reports indicate that simvastatin, cerivastatin and atorvastatin exert a biphasic effect on cultured ECs and angiogenesis in a dose-dependent manner.

“New generation” statins, which have a stronger effect on cholesterol reduction than conventional statins, have been developed and one of them, pitavastatin, exhibits a potent hypolipidemic effect in both animal and human trials. At a dose of 4 mg/day pitavastatin has been shown to be equivalent to atorvastatin at a dose of 80 mg/day in reducing low-density lipoprotein in heterozygous familial hypercholesterolemia patients. There are some reports of its pleiotropic effects on ECs; pitavastatin increased thrombomodulin, tissue-type plasminogen activator and nitric oxide production, and decreased plasminogen activator inhibitor 1 and inflammatory cytokine. Moreover, pitavastatin has augmented capillary formation in the ischemic limb of an animal model. These pleiotropic effects of pitavastatin are similar to those of conventional statins and so it is interesting to know whether or not the effect of pitavastatin on the migration, proliferation, and...
It is believed that statins exert a direct effect on ECs and angiogenesis by inactivating small GTP-binding protein, such as Rho, via inhibition of isoprenoid intermediates of the mevalonate pathway, including mevalonate (MEV), geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP). It has been demonstrated that inhibition of Rho by more than approximately 0.02 μmol/L of cerivastatin prevents organization of the actin cytoskeleton and, consequently, cell locomotion. In addition, it has been demonstrated to inactivate Akt, which is involved in the survival of ECs. In contrast, several studies have shown that less than 1 μmol/L of simvastatin activates the Akt dependent pathway. However, there has not been a study in which the molecular mechanism of the biphasic effect of statins on ECs and on angiogenesis has been observed. Moreover, it is unknown whether the discrepant effect of statins on ECs and angiogenesis is related to the peculiarity of each statin or the dosage used in each study.

Therefore, our study was designed to determine whether pitavastatin has a biphasic effect on ECs and on angiogenesis, as well as to clarify how this reagent affects the mevalonate pathway.

Methods

Cell Culture

Human epidermal microvessel endothelial cells (HMVECs) were purchased from Cambrex Co (Walkersville, MD, USA) and cultured in endothelial basal medium-2 (EBM-2) supplemented with hydrocortisone, vascular endothelial growth factor, ascorbic acid, gultamic acid, and amphotericin B, basic fibroblast growth factor (FGF)-2, insulin-like growth factor-1, heparin, epidermal growth factor, and 5% fetal bovine serum (FBS) (Cambrex Co). The cells at passage 4–15 were used for the experiments.

Scratch Assay

HMVECs were seeded at a density of 9.6×10⁴ in a 35-mm×6-well plate. When the cells achieved 70–80% confluence, half the cell area was scratched with a sterile cell scraper. After being washed with phosphate-buffered saline (PBS) twice, the cells were incubated for 48 h in EBM-2 containing 5% FBS, 25 ng/ml of FGF-2 (R&D systems, Minneapolis, MN, USA) in the presence of 0.01 μmol/L or 1 μmol/L pitavastatin (Kowa Co, Tokyo, Japan) or in the absence of pitavastatin (PBS only) as a control. We defined 0.01 μmol/L pitavastatin as low concentration because it is similar to the concentration achieved clinically with 2–8 mg pitavastatin by oral administration. Thus, 1 μmol/L was defined as high concentration. Cell migration into the scratched area was photographed at a magnification of ×40 after 24- 48-h incubation.

Cell Chemotaxis Assay

A cell chemotaxis assay was performed in a 48-well chemotaxis chamber (Neuro Probe Inc, Gaithersburg, MD, USA). HMVECs were preincubated for 24 h with the high or low concentration of pitavastatin. Then, 2×10⁴ of cell suspension was plated on the upper chamber of a transwell insert that included a polycarbonate membrane with 5 μm pores (Neuro Probe). The lower chamber was filled with EBM-2 medium containing 25 ng/ml of FGF-2. After 5-h incubation, all nonmigrating cells were removed from the upper face of the membrane with a rubber swab. The migrated cells on the lower surface of the membrane were fixed and stained with Diff-Quick stain (Kokusai Shiyaku, Kobe, Japan), and then the number of migrated HMVECs per high power field (magnification, ×200) was counted with an inverted phase-contrast microscope (Nikon, Tokyo, Japan). Five fields were selected in each experiment, and substituted for the average value of counting as an index of EC migration.

Cell Proliferation Assay

For quantification of cell proliferation, cells were incubated with 10 μmol/L bromodeoxyuridine (BrdU) for 4 h, and incorporated BrdU was detected by enzyme-linked immunosorbent assay according to the instructions of the manufacturer (Amersham Bioscience,). Briefly, 3×10³ cells in each well of a 96-well plate were cultured for 2 days. Then the high or low concentration of pitavastatin was added with 25 ng/ml of FGF-2 to each well. After 24-h incubation, BrdU was added to each well and the cells were incubated for a further 4 h. Absorbance at 450 nm was measured by a spectrophotometric microplate reader (Japan Inter Med, Tokyo, Japan).

Cell DNA Staining

To assess the effect of pitavastatin on cell death, we observed the DNA fragmentation of HMVECs using fluorochrome. After 24- and 48-h incubation with the indicated concentration of pitavastatin, HMVECs were stained with 10 μmol/L bisbenzimide (Hoecht 33258, Sigma, St Louis, MO, USA) for 2 h in the dark and observed under fluorescence microscopy (Nikon, Tokyo, Japan).

Trypan Blue Dye Exclusion Test

To quantitatively evaluate the effect of pitavastatin on cell viability, a trypan blue dye exclusion test was performed. Pitavastatin at the indicated concentration was added to 70–80% confluent HMVECs in a 90 mm plate, and incubated for up to 24 h. After incubation, the cells were detached with trypsin, and resuspended in 1 ml of EGM-2 containing bovin serum albumin. Next, trypan blue solution (Gibco, Grand Island, NY, USA) was added to the cell suspension at a final concentration of 0.2% and incubated for 1 min. The trypan blue positive cells were counted as dead cells using a hemocytometer under a light microscope (Nikon).

Assessment of Molecular Mechanism

To address whether inhibition of isoprenoid intermediates of cholesterol biosynthesis is involved in the pitavastatin effect, experiments for EC migration, proliferation and viability were also performed in the presence of MEV (100 μmol/L), GGPP (20 μmol/L) or FPP (20 μmol/L).

Angiogenesis Assay With Chorioallantoic Membrane (CAM) of Quail Eggs

A CAM assay was performed using fertilized quail eggs (embryonic days 0: E0) obtained from Hiroshima Laboratory Animals (Hiroshima, Japan) and a modification of the method of Parsons-Wingerter et al. Briefly, the eggs were maintained at 37°C for 56 h, then cracked and plated onto 35 mm dishes at E3 for culturing for a further 24 h at 37°C. At E4, 0.01, 0.05, 0.15, 0.3 or 1 μmol/L of pitavastatin in 500 μl of PBS was dropped onto each embryo in the presence of 1 μg of FGF-2. A CAM with 1 μg of FGF-2 and
without pitavastatin was defined as the control. After being incubated for 48 h at 37°C (E6), the embryos were fixed with 4% paraformaldehyde/2% gultaraldehyde (R&D Systems, Minneapolis, MN, USA) in PBS. After a 2-day fixation period at room temperature, the CAM was dissociated from the fixed embryo and mounted with polyvinyl alcohol (low viscosity, R&D Systems). The image of an arterial endpoint vessel from the middle region of the CAM was acquired at a resolution of 2,000 pixels/cm with a digital scanner (Epson, Tokyo, Japan). Five square regions (300×300 pixels) were selected from the digitized image, binarized to black and white, skeletonized and the total length of the vessels in each region were measured using the NIH Image 1.61 program.

Statistical Analysis
Significant values were determined using a two-tailed non-parametric Mann-Whitney U test. The results are expressed as mean values±standard errors of the mean (SEM); p<0.01 is considered as statistically significant.

Results

Scratch Assay (Fig 1)
After 24-h incubation, the high concentration of pitavastatin inhibited FGF-2-stimulated HMVEC migration, whereas the low concentration of pitavastatin enhanced it. When the examination was continued for 48 h, the difference between the low and high concentrations became more marked. At the high concentration, pitavastatin also
induced detachment and morphologic change of HMVECs.

Chemotaxis Assay
In order to confirm the result of the scratch assay, we measured the chemotaxis of HMVECs against FGF-2 using a 48-well chemotaxis chamber containing an insert filter. HMVECs were pre-incubated with the indicated concentration of pitavastatin, and then placed onto the upper chamber. After 5-h incubation in the chemotaxis chamber, the high concentration of pitavastatin showed a significant (p<0.01) inhibition of HMVEC migration up to –44.2±5.3% compared with the control, but the low concentration increased the migration up to +33.3±13.8% vs the control (Fig 2). Cotreatment of ECs with MEV, GGPP or FPP did not reverse the inhibitory effect of the high concentration of pitavastatin. In contrast, the promotional effect of the low concentration was reversed with MEV, GGPP and FPP, but not with FPP.

Cell Proliferation Assay
The low concentration of pitavastatin enhanced FGF-2 induced cell proliferation (+28.7±24.1% vs control), whereas the high concentration significantly inhibited cell proliferation up to –26.5±6.4% compared with the control (p<0.01, Fig 3). Although the productive effect of the low concentration was reversed with MEV, GGPP and FPP, they did not cancel the effect of the high concentration.

Cell DNA Staining
The high concentration of pitavastatin increased the number of dead cells, which showed nuclear shrinkage and condensation after 24-h incubation (Fig 4). Further incubation for 24 h with the high concentration apparently promoted the cell death of HMVECs. MEV and GGPP but not FPP completely reversed the toxic effect of the high concentration of pitavastatin (Fig 5). The low concentration of pitavastatin did not obviously change the shape of the nuclei.

Trypan Blue Dye Exclusion Test (Fig 6)
Trypan blue positive cells increased in the presence of the high concentration of pitavastatin compared with control at 24h (9.79±0.86% for control and 15.47±0.76% for high concentration), whereas the low concentration significantly reduced the number of trypan blue positive cells (–28.7±8.16% vs control, p<0.01). The reduction of cell viability induced by the high concentration was abolished by MEV or GGPP but not by FPP. The cell-protective effect of the low concentration tended to be reversed by the isoprenoid products.
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CAM Assay (Fig 7)
The formation of new blood vessels on the CAM was observed in the PBS group (Fig 7A-a), and FGF-2 enhanced vascular development of arterioles (Fig 7A-b). When we tested 0.01, 0.05, 0.15, 0.3 or 1 μmol/L of pitavastatin in 500 μL of PBS, 0.15 (data not shown) and 0.3 μmol/L (Fig 7A-c) of pitavastatin increased, but 1 μmol/L decreased vascular formation of the CAM (Fig 7A-d). In the quantitative analysis, the formation of vascular structures in the CAM was strongly enhanced by FGF-2 (+30.7±33.2% to PBS only). High concentration (1 μmol/L) of pitavastatin significantly reduced FGF-2-induced angiogenesis (–25.9±21.0% vs control), whereas low concentration (0.3 μmol/L) tended to increase FGF-2-induced angiogenesis (+11.9±20.6% vs control) (Fig 7B).

Discussion
In the present study, we demonstrated that pitavastatin has a biphasic effect on ECs and angiogenesis similar to other statins.25,27 Pitavastatin at the high concentration inhibited migration and proliferation of ECs and increased cell death, whereas the low concentration of pitavastatin protected cell viability, and enhanced migration and proliferation. Although the cell death induced by the high concentration was reversed by MEV or GGPP, but not by FPP, the statin’s inhibitory effects on migration and proliferation were not cancelled. The cell-activating effect of the low concentration was reversed by MEV, GGPP or FPP.

In our experiments, we used HMVECs originating from adult human microcapillaries. Our experimental results can be applied to macrovasculature original ECs, such as human umbilical vascular endothelial cells (HUVECs) or bovine aortic endothelial cells, because a biphasic effect of atorvastatin on migration, proliferation and cell death has been demonstrated using HUVECs27 in addition to HMVECs.25

Kureishi et al demonstrated that 1 μmol/L of simvastatin activated ECs and angiogenesis via activation of Akt-dependent NO synthesis.20 Although the mediator of Akt is not completely understood, Rorowski et al argue that the inhibition of cholesterol biosynthesis in ECs by statins will alter the cholesterol concentrations within lipid rafts, which...
are specific membrane domains and are implicated in the regulation of MAPK and PI3K, leading to recruitment or retention at these sites and subsequent activation of Akt. Furthermore, it is well established that statins increase eNOS expression via Rho inactivation. Therefore, supplementation of FPP, which is a precursor of cholesterol, may inactivate Akt through the cholesterol biosynthesis pathway, and supplementation of GGPP may activate Rho though the non-cholesterol biosynthesis pathway. Taking all the findings together, a low concentration of statin may affect both the cholesterol-dependent Akt and cholesterol-independent Rho pathways.

In contrast, several studies have demonstrated that statins inhibit migration and proliferation of ECs and induce cell death at high concentration by inhibiting the activation of Rho. Those reports, as well as our own results for cell death, demonstrate that these inhibitory effects are reversed by MEV or GGPP but not by FPP. In order to exert its role in cell signal transduction, Rho must translocate from the cell cytoplasm to the cell membrane. As GGPP, but not FPP, is essential for this process, our result supports the theory that the inhibitory effects of statins depend on Rho inactivation. Laufs et al propose that inactivation of Rho increases NO synthesis, which may produce a beneficial effect. However, there are some reports of harmful oxidant derived from NO and subsequent impaired endothelial function. Thus, a high concentration of statin may impair ECs by excessive NO synthesis via potent Rho inhibition.

Weis et al demonstrated that the inhibitory effects of...
atorvastatin and cerivastatin on EC proliferation, migration and viability were reversed by MEV or GGPP$^{25}$ and our data concur with them regarding viability, but not for proliferation and migration, which suggests that a high concentration of pitavastatin may inhibit the proliferation and migration of ECs via an unknown route beyond the mevalonate pathway.

In the present study, we demonstrated that a high concentration of pitavastatin (1 μmol/L) interrupted the development of FGF-2-induced vasculature in the CAM, whereas the low concentration of pitavastatin (0.3 μmol/L) tended to enhance it. In the CAM assay, the pro-angiogenic effect of pitavastatin was unexpectedly observed at a higher concentration than in the experiments using cultured HMVECs. It is possible that pitavastatin is not completely absorbed by eggs and, therefore, in a CAM assay the actual concentration of pitavastatin may be much lower than the indicative concentration. Thus, the activating effect on neovascularization of different concentrations of pitavastatin may be observed between experiments using cultured HMVECs and those using a CAM assay.

The direct effects of statins on ECs and angiogenesis have been reported by a number of investigators. Kuroishi et al demonstrated that 1 μmol/L of simvastatin promoted EC survival$^{20}$ whereas Vincent et al showed that approximately 0.02–0.05 μmol/L (10–25 ng/ml) of cerivastatin inhibited...
EC migration and in vivo angiogenesis. Later, Weis et al. demonstrated that low concentrations (0.005–0.05 μmol/L) of cerivastatin and atorvastatin promoted proliferation and migration of ECs, whereas an inhibitory effect was observed at higher concentrations (>0.05 μmol/L). On the basis of these previous studies, and the results of the present study, it would seem that the type of statin as well as its concentration may be important factors in the response of ECs. Clinically, the serum level of cerivastatin in humans is between 0.002 and 0.05 μmol/L when 0.2–0.8 mg/day is taken, and for atorvastatin the level is between 0.002 and 0.2 μmol/L when 10–80 mg/day is taken. The respective serum level of simvastatin is less than 0.09 μmol/L when 40 mg/day is taken.25 Cerivastatin, atorvastatin and pitavastatin exert a protective effect on ECs at low concentrations in humans. Although higher doses of simvastatin (>10 μmol/L) decrease cell viability, Kureishi et al. showed that simvastatin exerted a protective effect on ECs even above a clinically relevant dose25 which is inconsistent with Weis’s and our own results. This discrepancy may be attributable to the characteristics of each statin.

Statins also influence endothelial progenitor cells (EPCs), which are known to contribute to vasculogenesis, as well as ECs.26–28 Llevadot et al. demonstrated that 0.1–10 μmol/L of simvastatin promoted EPC proliferation, migration, mobilization from bone marrow, and cell survival in vitro via the Akt signaling pathway.29 Urbich et al. reported that atorvastatin induced a dose-dependent increase in EPC migration with a maximal effect at 0.01 μmol/L, and that preincubation of EPCs for 24h with 0.01 μmol/L atorvastatin stimulated in vitro tube formation by EPCs.29 It is therefore possible that pitavastatin directly affects induction, migration and proliferation of EPCs in the CAM vasculature, but it is not clear whether pitavastatin influenced angiogenesis or vasculogenesis because the effect of pitavastatin on EPCs were not assessed in the present study.

With respect to the effect of statins on angiogenesis in animal trials, Kureishi et al. demonstrated that 0.1 mg·kg−1·day−1 of simvastatin induced angiogenesis in the ischemic limbs of normocholesterolemic rabbits29 and a similar result has been demonstrated with a high dose (6 mg·kg−1·day−1) of cerivastatin in ischemic mice.30 Weis et al. reported that although high doses (2.5 mg·kg−1·day−1) of cerivastatin inhibited the growth and angiogenesis of lung tumors, low doses (0.5 mg·kg−1·day−1) did not promote tumor growth.7 Moreover, they demonstrated that, with regard to inflammatory angiogenesis, low doses of both cerivastatin and atorvastatin (0.5 mg·kg−1·day−1) enhanced vascular formation in a subcutaneous implanted disk whereas high doses of cerivastatin (2.5 mg·kg−1·day−1) inhibited it.25 On the basis of the previous research and our own analysis, a clinical dose of statins seems to increase angiogenesis, except for tumor growth, although it is still unclear whether or not a high dose inhibits it.

Angiogenesis is essential to various pathophysiological phenomena such as tumor growth, inflammation, and collateral vessel formation in ischemic tissue, and it may be, therefore, a beneficial therapeutic target. In the present study, a low concentration of pitavastatin enhanced angiogenesis, indicating that ordinary doses of statins may assist angiogenic therapy of ischemic diseases such as arteriosclerosis obliterans. On the other hand, it may be advisable to promote careful statin use for patients who have angiogenic diseases such as malignancy and diabetic retinopathy, although statins have not increased the morbidity of malignancy in clinical trials.25

In this study, a high concentration of pitavastatin inhibited FGF-2-induced angiogenesis, suggesting the potential of pitavastatin as a therapeutic agent against angiogenic diseases. Because the concentrations used in the present study were much higher than the maximum clinical serum level in humans, such a situation would not exist clinically when statin is taken orally. Therefore, if pitavastatin is to be used as an anti-angiogenic therapy, it would have to be injected directly into the lesion.

**Conclusion**

Pitavastatin has a biphasic effect on ECs and angiogenesis. A low concentration (0.01 μmol/L) may exert its pro-angiogenic effect via both cholesterol-dependent and -independent pathways. A high concentration (1 μmol/L) may inhibit angiogenesis via an unknown route in addition to the conventional pathway. These results suggest that pitavastatin is a potential therapeutic agent for ischemic diseases, such as arteriosclerosis obliterans, at low doses and for angiogenic diseases, such as malignancy and inflammation, at high doses.

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