

Pravastatin-induced proangiogenic effects depend upon extracellular FGF-2

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Received: May 26, 2011; Accepted: November 2, 2011

Abstract

The HMG-CoA reductase inhibitors (statins) have been shown to exert several protective effects on the vasculature that are unrelated to changes in the cholesterol profile, and to induce angiogenesis. The proangiogenic effect exerted by statins has been attributed to the activation of the PI3K/Akt pathway in endothelial cells; however, it is unclear how statins activate this pathway. Pravastatin-mediated activation of Akt and MAPK occurs rapidly (within 10 min.) and at low doses (10 nM). Here, we hypothesized that FGF-2 contributes to the proangiogenic effect of statins. We found that pravastatin, a hydrophilic statin, induced phosphorylation of the FGF receptor (FGFR) in human umbilical vein endothelial cells. SU5402, an inhibitor of FGFR, abolished pravastatin-induced PI3K/Akt and MAPK activity. Likewise, anti-FGF-2 function-blocking antibodies inhibited Akt and MAPK activity. Moreover, depletion of extracellular FGF-2 by heparin prevented pravastatin-induced phosphorylation of Akt and MAPK. Treatment with FGF-2 antibody inhibited pravastatin-enhanced endothelial cell proliferation, migration and tube formation. These observations indicate that pravastatin exerts proangiogenic effects in endothelial cells depending upon the extracellular FGF-2.

Keywords: statin • pleiotropic effect • endothelial cells • FGF-2

Introduction

The HMG-CoA reductase inhibitors known as statins are potent blockers of cholesterol biosynthesis and are widely used in the treatment of hypercholesterolaemia [1, 2]. Substantial experimental and clinical evidence indicates that the beneficial effects of statins extend beyond cholesterol reduction and involve improved plaque composition, decreased inflammation and ameliorated endothelial functions [2, 3]. All statins may have the potential to promote angiogenesis, resulting in an increase in capillary density and perfusion rates in the microvasculature,

and thereby contributing to the restoration of vascular homeostasis. These activities have been demonstrated in animal models of ischaemia, particularly in a mouse ischaemic limb [4] and in rabbit hindlimb ischaemia [5]. *In vitro* studies using endothelial cells (ECs) have revealed that low concentrations of statins promote proliferation, migration and formation of capillary-like structures [6, 7]. A potential mechanism by which statins may improve endothelial function and promote angiogenesis is through their activation of the PI3K/Akt pathway [2, 5]. Statin treatment induces rapid Akt-mediated phosphorylation of endothelial-derived nitric oxide synthase (eNOS), which results in nitric oxide production [5]. Simvastatin-mediated Akt activation occurs rapidly (within 15 min.) and at low doses (0.1 μ M) [5]. There is considerable evidence that activation of the PI3K/Akt pathway may contribute to the endothelium-dependent effects of statins, but the precise mechanisms by which PI3K is activated have not yet been identified. Additionally, it has been reported that atorvastatin enhanced phosphorylation of MAPK (ERK1,2) as well as Akt in mesenteric vessels of the

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spontaneously hypertensive rat [8]. Lipophilic statins, such as simvastatin and atorvastatin, are considered more likely to enter ECs by passive diffusion than hydrophilic statins, such as pravastatin, which are targeted to the liver. It would also seem that of all the statins, pravastatin has the lowest potency to inhibit HMG-CoA reductase in cultured human ECs [9]. However, in cultured ECs, pravastatin was at least as effective as more lipophilic statins [5, 10] at stimulating the release of nitric oxide. In fact, our previous research showed that pravastatin induced eNOS phosphorylation within 5 min. in human umbilical vein endothelial cells (HUVECs) [4, 11]. Thus, the lipophilicity within ECs does not entirely predict the ability of statins to improve EC functions, and hence, other unidentified factors may play a role in this function. Hydrophilic statins might transmit the signal to ECs *via* specific mechanisms. Hence, we hypothesized that statins may exert beneficial potency to endothelial functions *via* the activation of receptors on ECs.

Angiogenesis is regulated by many proangiogenic growth factors, including VEGF, FGF-2 and angiopoietin-1 [12–14]. Among these, FGF-2 facilitates angiogenesis *via* the activation of both the MAPK and PI3K/Akt pathways. FGF-2, a member of the heparin-binding growth factors, has multifunctional actions such as promoting cell survival in many cell types and affecting differentiation and gene expression. Accordingly, we investigated the hypothesis that the activation of the FGFR is a key step in the activation of intracellular signalling for angiogenesis.

In this research, we investigated the phosphorylation of FGFR on cells exposed to pravastatin. Furthermore, we examined the activation of ERK1,2 and Akt in cells exposed to pravastatin when FGFR was pharmacologically blocked. We evaluated the role of extracellular FGF-2 in pravastatin-induced phosphorylation of MAPK and Akt. We also studied the influence of inhibition of extracellular FGF-2 by a function-blocking antibody on endothelial proliferation, migration and tube formation. Here, we report on the activation of the FGF-2/FGFR cascade, which plays a central role in the proangiogenic effects of statins.

Materials and methods

Materials

Pravastatin was provided by DAIICHI SANKYO Co., Ltd. Simvastatin was provided by Merck Co., Ltd., and pitavastatin was provided by KOWA PHARMACEUTICAL Co., Ltd. Recombinant FGF-2 and SU5402 were obtained from R&D (R&D Systems, Minneapolis, MN, USA) and Merck (Darmstadt, Germany). The anti-FGF-2 (neutralizing) antibody (bFM-1) was from Millipore (Temecula, CA, USA). Antibodies were obtained from the following sources: anti-Akt (p-Ser473), Akt1, ERK1,2 (p-Thr202/p-Tyr204), ERK1,2 and anti-eNOS (p-Ser1177) antibodies from Cell Signaling (Danvers, MA, USA); anti-FGF-2 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-FGFR (p-Tyr653/p-Tyr654) antibody from R&D Systems; and anti- β -actin antibody from Sigma-Aldrich (St. Louis, MO, USA). The anti- α -tubulin antibody was obtained from Oncogene (San Diego,

CA, USA). The horseradish peroxidase (HRP)-coupled secondary antibodies were from GE Healthcare (Uppsala, Sweden). The heparin was purchased from Sigma-Aldrich.

Cell culture

EGM2 and EBM2 culture media were purchased from Lonza (Walkersville, MD, USA). HUVECs (passage 5 or less) were obtained from Lonza and grown at 37°C in a humidified atmosphere of 5% CO₂ in EGM2 medium. Foetal bovine heart endothelial (FBHE) cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). Cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 2 ng/ml recombinant FGF-2 (WAKO, Osaka, Japan) at 37°C in a 5% CO₂ incubator. All procedures were in accordance with institutional guidelines for animal research.

Immunoblotting

Our detailed method has been described previously [15]. Cells were washed with phosphate buffered saline (PBS) and lysed in RIPA lysis buffer consisting of 50 mM HEPES, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% CHAPS, 10% glycerol, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Lysates were clarified by centrifugation, the supernatant was recovered and protein concentrations were assayed using the bicinchoninic acid protein assay reagent (Thermo Fisher Scientific, Rockford, IL, USA). Lysates for immunoblotting (25 μ g of protein) were separated on SDS-polyacrylamide gels under reducing conditions, followed by electrophoretic transfer to polyvinylidene difluoride membranes (Immobilon-P; Millipore). After blocking, the membranes were probed with the appropriate primary antibodies. Membrane-bound primary antibodies were detected using secondary antibodies conjugated with HRP. Immunoblots were detected with LAS-3000 (FUJIFILM, Tokyo, Japan) using the enhanced chemiluminescence technique (Immobilon Western HRP Substrate; Millipore). Immunoblots were quantified using Image Gauge software (FUJIFILM).

Heparin wash and ELISA

HUVECs were starved by culturing in EBM2 for 18 hrs. The media was removed and cells were incubated with or without heparin (10 μ g/ml) in PBS for 20 min. at room temperature on a rocking shaker. Cells were treated with pravastatin for 10 min. The heparin solution (50 μ l) was removed, and the level of FGF-2 was determined using the Human FGF basic ELISA kit (R&D Systems) according to the manufacturer's instructions.

Proliferation assay

FBHE cells (1×10^4 cells) were seeded into 6-well plates and cultured with DMEM:RPMI 1640 (1:1) supplemented with 2% FBS and 0.1 μ g/ml heparin for 24 hrs. Thereafter, pravastatin (1 μ M) and/or FGF-2-neutralizing antibodies (2.5 μ g/ml) were added to the cells for 4 days. Cells were then trypsinized and counted using a Coulter counter (Beckman-Coulter, Brea, CA, USA).

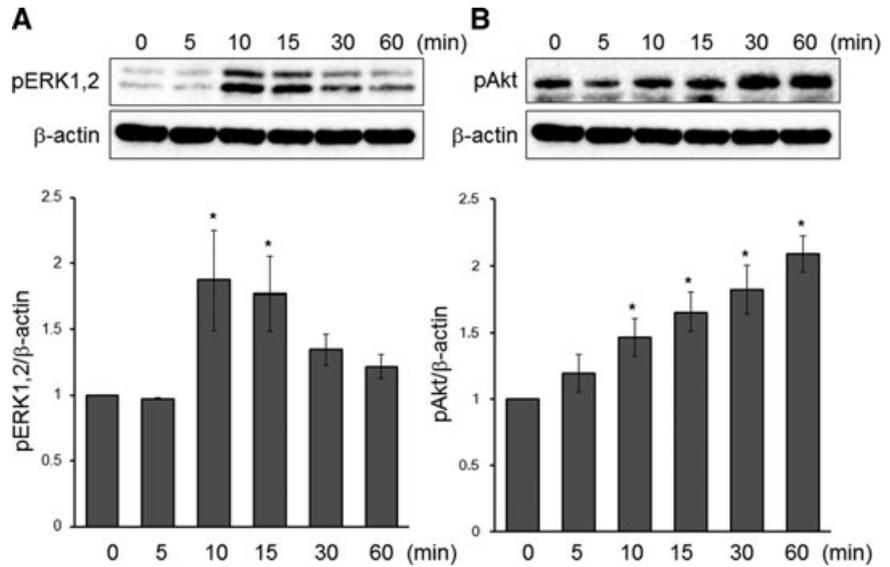


Fig. 1 Pravastatin induces phosphorylation of ERK1,2 and Akt. HUVECs were incubated in EBM2 for 12 hrs and then stimulated with pravastatin (1 μ M) for the indicated times. The lower panels indicate densitometric quantitation of each protein. (A) ERK1,2, $N = 3$; (B) Akt, $N = 5$; * $P < 0.05$ versus 0 min.

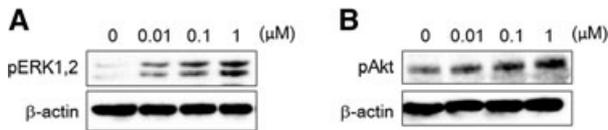


Fig. 2 Pravastatin induces phosphorylation of ERK1,2 and Akt in a dose-dependent manner. HUVECs were incubated in EBM2 for 12 hrs and then stimulated with pravastatin (0.01–1 μ M) for 10 min. (A) ERK1,2; (B) Akt.

Statistical analysis

All data are presented as means \pm S.E. Comparisons among groups were made by one-way ANOVA. For differences between two groups, Student's *t*-test was used when appropriate. Differences were considered statistically significant at a value of $P < 0.05$.

Migration assay

Transwell inserts (Nunc, Rochester, NY, USA) were coated with 0.1 mg/ml collagen. Pravastatin at 1 μ M dissolved in EBM-2 medium containing 0.25% bovine serum albumin (BSA) was added to the bottom chamber of the Boyden apparatus. HUVECs (1×10^5 cells) suspended in 100 μ l aliquot of EBM2 containing 0.25% BSA were added to the upper chamber. After a 6-hr incubation, the non-migrating cells in the upper part of the chamber were mechanically removed, and the remaining cells in the lower part were fixed with methanol. For quantification, cells were stained by Diff-Quik solutions (Kokusai Shiyaku, Kobe, Japan). Cells migrating into the lower chamber were counted in six random microscopic fields.

Tube formation assay

The formation of vascular-like structures by HUVECs was assessed on growth factor-reduced Matrigel (BD Biosciences, Bedford, MA, USA) [15]. HUVECs were plated at 1×10^4 cells/well in a 96-well-multidish precoated with 30 μ l of Matrigel in the absence or presence of pravastatin (1 μ M). After 6 hrs of incubation in a 5% CO₂-humidified atmosphere at 37°C, the cells were stained by Calcein-AM (5 μ g/ml). Then, the lengths of completed tube-like structures in a centre field (10-fold magnification) were quantified. Images were captured with a CCD colour camera attached to the microscope, and tube length was measured using a Micro Analyzer (JPD, Tokyo, Japan).

Results

Pravastatin-activated ERK1,2 and Akt pathway

Previous reports have suggested that several statins activate the MAPK and PI3K/Akt pathways [5, 16]. To evaluate the direct effects of pravastatin on ECs, we first investigated the effects of pravastatin on the time-dependent phosphorylation of ERK1,2 and Akt. In all experiments, ECs were treated with pravastatin at a dosage of 1 μ M, which is the most effective dose in rat EC proliferation [17]. As shown in Figure 1A, addition of pravastatin (1 μ M) to HUVECs resulted in transient phosphorylation of ERK1,2 (Fig. 1A). We next tested the effect of pravastatin on the phosphorylation of Akt. Akt was significantly phosphorylated in the presence of pravastatin within 10 min. and phosphorylation gradually increased for 1 hr (Fig. 1B). Since both ERK1,2 and Akt were phosphorylated by pravastatin at 10 min., we performed all subsequent assessments of phosphorylation at 10 min. The same was true when the cells were treated with pravastatin at 100 nM and 10 nM (Fig. 2). Although pravastatin is hydrophilic, it could phosphorylate ERK1,2 and Akt rapidly and at low concentration (10 nM). These data suggest that pravastatin might activate signalling through a receptor rather than by penetrating a cell membrane.

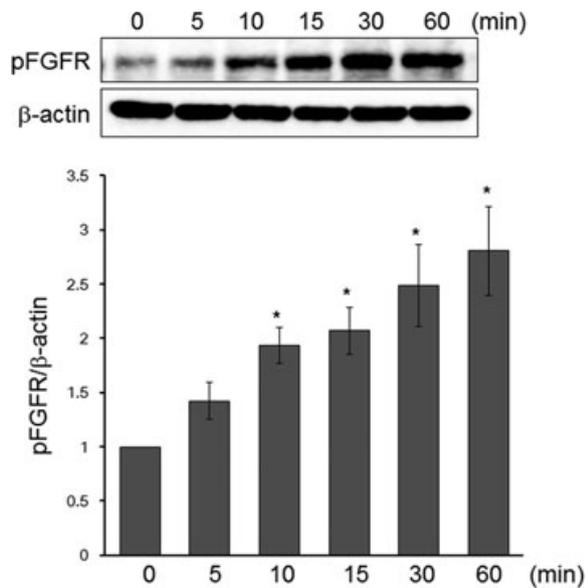


Fig. 3 FGFR phosphorylation is induced by pravastatin. Quiescent HUVECs were starved for 12 hrs and then treated with pravastatin (1 μ M) for 5 min. to 1 hr. The cells were lysed and analysed for phospho-FGFR protein by immunoblotting. The lower panels indicate the densitometric quantitation results of immunoblots. $N = 7$, * $P < 0.05$ versus 0 min.

Pravastatin-phosphorylated FGFR

HUVECs were stimulated with pravastatin for 5 min. to 1 hr. FGFR phosphorylation was detected as early as 10 min. after pravastatin

stimulation, and FGFR remained phosphorylated after 1 hr (Fig. 3). The temporal patterns of the effects of pravastatin on FGFR phosphorylation were similar to those on Akt phosphorylation (Fig. 1B).

Participation of FGF-2/FGFR in pravastatin-induced activation of ERK1,2 and Akt

The stimulation of FGFR by pravastatin involves the sequential activation of ERK1,2 and Akt. To determine whether the activation of the MAPK and PI3K/Akt pathways by pravastatin depends on FGFR phosphorylation, quiescent HUVECs were stimulated with pravastatin in the absence or the presence of SU5402 (10 μ M), the FGFR tyrosine kinase inhibitor. Pravastatin induced ERK1,2 activation at 10 min., whereas pretreatment of ECs with SU5402 completely inhibited pravastatin-induced ERK1,2 phosphorylation (Fig. 4A). Preincubation of ECs with SU5402 alone did not affect the activation of ERK1,2. Likewise, evaluation of the effects of FGFR on Akt activation demonstrated that pravastatin-induced phosphorylation of Akt was suppressed by SU5402 (Fig. 4B). Next, we examined the participation of FGF-2 in FGFR activation by blocking extracellular FGF-2. The effects of FGF-2 on ERK1,2 and Akt activation was suppressed by FGF-2 antibody. Preincubation of ECs with FGF-2-neutralizing antibody (5 μ g/ml) for 1 hr showed that the phosphorylation of ERK1,2 and Akt induced by pravastatin was comparable to that suppressed by SU5402 (Fig. 5A and B). Preincubation of ECs with SU5402 or FGF-2 antibody did not affect ERK1,2 and Akt phosphorylation. Thus, we found that the FGF-2/FGFR system contributes to pravastatin-induced ERK1,2 and Akt activation.

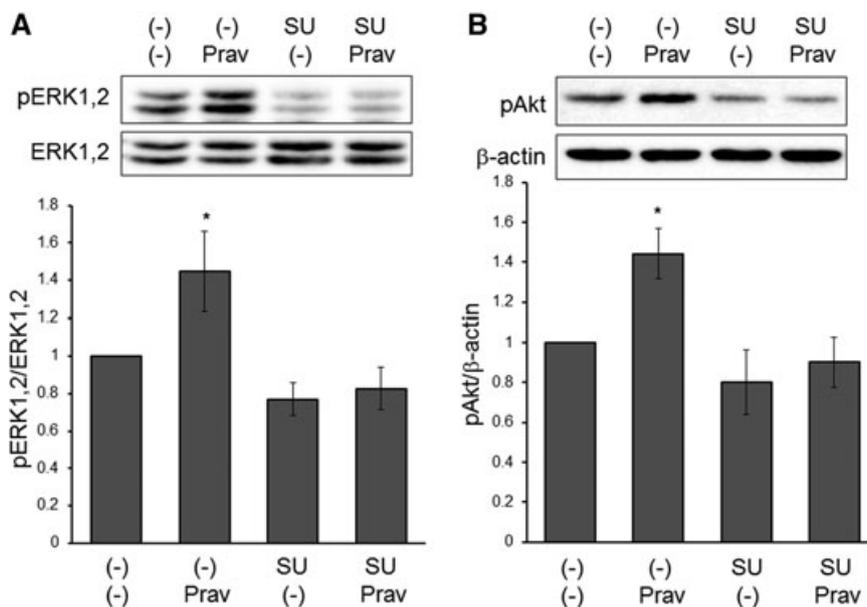


Fig. 4 ERK1,2 or Akt phosphorylation requires FGFR activation. HUVECs were preincubated with SU5402 for 1 hr, and then stimulated with pravastatin for 10 min. The phosphorylation of ERK1,2 (A) or Akt (B) was analysed by immunoblotting. $N = 3$, * $P < 0.05$ versus (-). Prav: pravastatin; SU: SU5402.

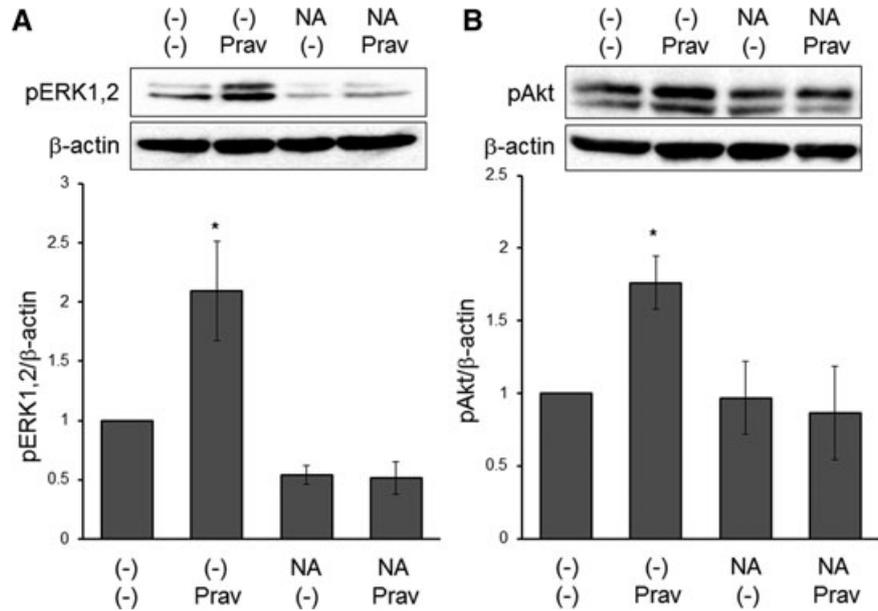


Fig. 5 ERK1,2 or Akt phosphorylation requires extracellular FGF-2. HUVECs were preincubated with FGF-2-neutralizing antibody for 1 hr, and then stimulated with pravastatin for 10 min. The phosphorylation of ERK1,2 (A) or Akt (B) was analysed by immunoblotting. *N* = 3, **P* < 0.05 versus (-). Prav: pravastatin; NA: neutralizing antibody.

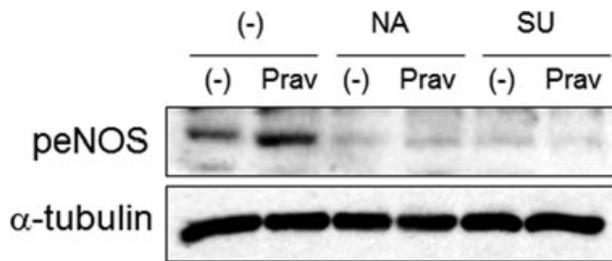


Fig. 6 eNOS phosphorylation requires the FGF-2/FGFR system. HUVECs were preincubated with FGF-2-neutralizing antibody or SU5402 for 1 hr, and then stimulated with pravastatin for 10 min. The phosphorylation of eNOS was analysed by immunoblotting. Results were similar in two other experiments. Prav: pravastatin; NA: neutralizing antibody; SU: SU5402.

FGF-2-neutralizing antibody and SU5402-inhibited pravastatin-induced eNOS phosphorylation

Statins induce Akt-mediated phosphorylation of eNOS at Ser1177, which results in nitric oxide production, but with no accompanying change in the total level of eNOS protein. To determine whether the activation of the eNOS by pravastatin depends on FGFR phosphorylation, quiescent HUVECs were stimulated with pravastatin in the absence or the presence of FGF-2-neutralizing antibody (5 μg/ml) or SU5402 (10 μM). Pravastatin induced eNOS phosphorylation at 10 min., whereas pretreatment of ECs with FGF-2-neutralizing antibody or SU5402 completely inhibited pravastatin-induced eNOS phosphorylation (Fig. 6).

Pravastatin activates ERK1,2 and Akt via extracellular FGF-2

The inhibitory effect of the FGF-2 antibody on the signalling pathway suggests that extracellular FGF-2 functions as a trigger in pravastatin signalling. To determine the extracellular FGF-2-mediated effects of pravastatin, the cell layer was washed with heparin to remove any FGF-2 bound to the cell membrane or extracellular matrix, as previously described [18]. The amount of FGF-2 in the heparin wash solution was measured by ELISA. The cell layer was washed after a 20-min. incubation with heparin and yielded 18 ng of extracellular FGF-2 (Fig. 7A). After heparin washing, ECs were stimulated with pravastatin for 10 min. Phosphorylation of ERK1,2 and Akt was inhibited by the heparin wash, but not by the PBS wash (Fig. 7B and C). Hence, depletion of extracellular FGF-2 affected intracellular signalling by pravastatin. No significant differences were found in ERK1,2 activation or in Akt phosphorylation after the heparin wash (Fig. 7B and C). There were no differences in the levels of phospho-ERK1,2 and phospho-Akt between the control in PBS and control in heparin wash. These results suggest that the activation of MAPK and PI3K/Akt pathway by pravastatin depends on extracellular FGF-2.

Simvastatin or pitavastatin-induced ERK1,2 phosphorylation requires extracellular FGF-2

To determine whether the activation of the MAPK and PI3K/Akt pathways induced by other statins depends on extracellular FGF-2, quiescent HUVECs were stimulated with simvastatin or pitavastatin in the absence or the presence of FGF-2-neutralizing antibody

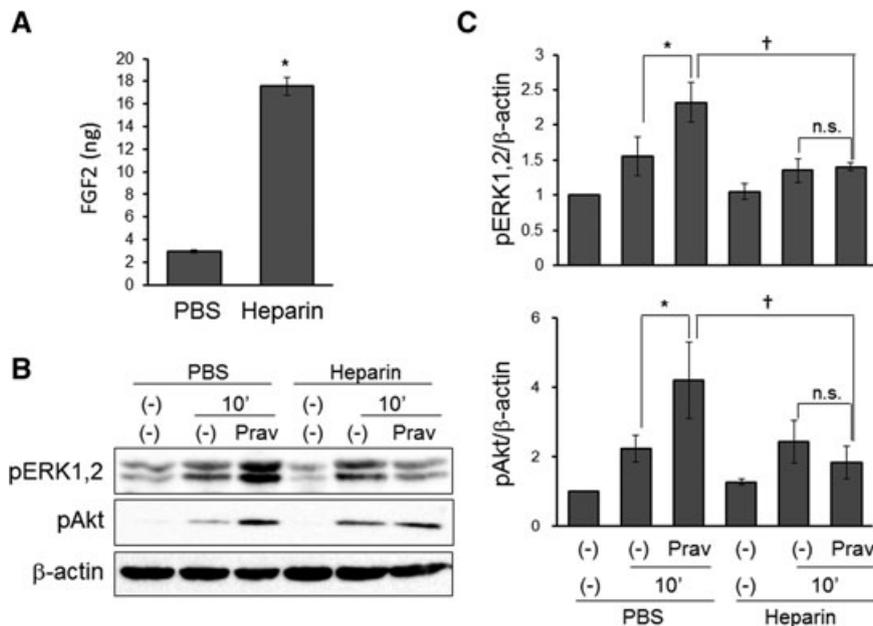


Fig. 7 Pravastatin-induced ERK1,2 and Akt are modulated by extracellular FGF-2. **(A)** FGF-2 was removed from the cell layer by washing with heparin-containing PBS and quantified by ELISA. $N = 5$, $*P < 0.05$ versus PBS. **(B, C)** After washing with heparinized PBS, the cells were treated with pravastatin (1 μM) for 10 min., and then ERK1,2 and Akt activity was measured. **(C)** indicates the quantitative data of **(B)**. $N = 5$, $*P < 0.05$ versus PBS wash and (-) 10 min. $^{\dagger}P < 0.05$ versus heparin wash and pravastatin 10 min.

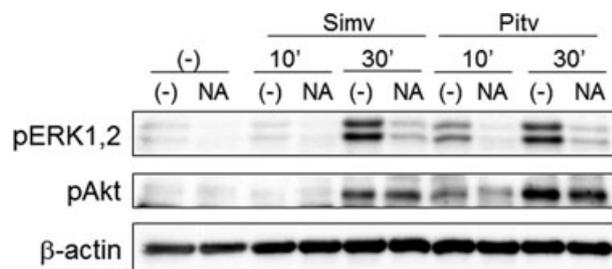


Fig. 8 Simvastatin or pitavastatin-induced ERK1,2 or Akt phosphorylation requires extracellular FGF-2. HUVECs were preincubated with FGF-2-neutralizing antibody for 1 hr, and then stimulated with simvastatin (100 nM) or pitavastatin (100 nM) for 10 min. or 30 min. The phosphorylation of ERK1,2 or Akt was analysed by immunoblotting. Data are representative of two independent experiments that yielded similar results. Simv: simvastatin; Pitv: pitavastatin; NA: neutralizing antibody.

(5 $\mu\text{g/ml}$). Both simvastatin and pitavastatin induced ERK1,2 activation at 30 min., whereas pretreatment of ECs with FGF-2-neutralizing antibody significantly inhibited statin-induced ERK1,2 phosphorylation (Fig. 8). Pitavastatin-induced phosphorylation of Akt was partially suppressed by FGF-2-neutralizing antibody, whereas simvastatin-induced Akt phosphorylation was not inhibited by FGF-2-neutralizing antibody.

Pravastatin-induced EC proliferation *via* FGF-2

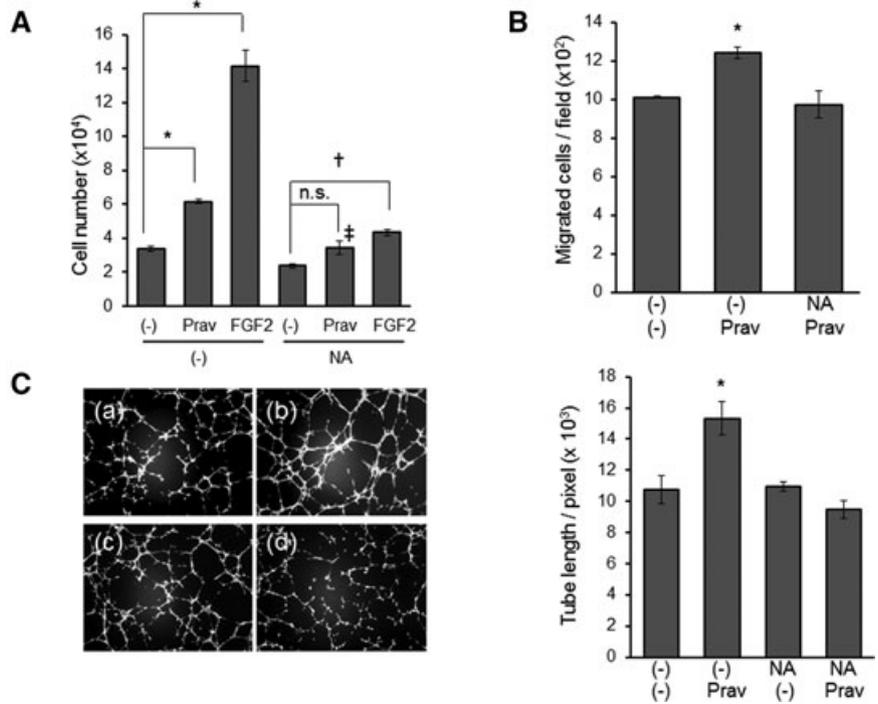
To elucidate the contribution of extracellular FGF-2 to the pleiotropic effects of pravastatin, we investigated the effects of

pravastatin treatment on angiogenesis *in vitro*. In our previous study, 1 μM of pravastatin increased rat aortic EC proliferation by 20% [17]. Thus, we investigated whether pro-proliferative actions of pravastatin might depend on the availability of endogenous FGF-2. FBHE cells are dependent on FGF-2 for survival and proliferation [19]. FBHE cells were incubated with 1 μM pravastatin in the presence or absence of FGF-2 antibody (2.5 $\mu\text{g/ml}$) for 4 days, and proliferation was assessed. Pravastatin induced a 2-fold increase in cell number, while 1 ng/ml of FGF-2 (positive control) induced a 5-fold increase in cell number (Fig. 9A). However, treatment with FGF-2-neutralizing antibody significantly inhibited the pravastatin-induced proliferative effect. In a control experiment, FGF-2 antibody inhibited proliferation in response to FGF-2; however, FGF-2 antibody did not induce cell death. Therefore, the pravastatin proliferative action is dependent on the availability of extracellular FGF-2.

Pravastatin-induced EC migration *via* FGF-2

Because EC migration is known to be an important event in angiogenesis, we further investigated the role of extracellular FGF-2 in pravastatin-induced migration of HUVECs. HUVECs were placed into transwells in the absence or presence of FGF-2 antibody (2.5 $\mu\text{g/ml}$). Pravastatin caused a 1.2-fold increase in the number of cells migrating into the lower chamber, and the effect of pravastatin was significantly suppressed by the addition of FGF-2 antibody (Fig. 9B). These results demonstrate that the presence of FGF-2 is necessary to stimulate EC migration in response to pravastatin.

Fig. 9 Inhibition of extracellular FGF-2 suppressed pravastatin-enhanced endothelial function. **(A)** Proliferation: FBHE (1×10^4) cells were cultured in 2% FBS-DMEM/RPMI 1640 (1:1) for 24 hrs. The cells were then cultured with the vehicle, pravastatin (1 μ M) or FGF-2 (1 ng/ml) in the presence or absence of the FGF-2-neutralizing antibody (2.5 μ g/ml) for 4 days. The cell number was determined using a Coulter counter. $N = 3$, * $P < 0.05$ versus (-)/(-), † $P < 0.05$ versus (-)/NA, ‡ $P < 0.05$ versus Prav/(-). **(B)** Migration: cell migration was measured using transwell assays. HUVECs (1×10^5) were added to the upper well of the transwell insert in the absence or presence of FGF-2-neutralizing antibody (5 μ g/ml). After 6 hrs, the number of cells that had migrated to the lower surface of the membrane was counted. $N = 3$, * $P < 0.05$ versus (-)/(-). **(C)** Tube formation: HUVECs were seeded in 96-well plates coated with growth factor-reduced Matrigel. Cells were then treated with the vehicle or pravastatin in the presence or absence of FGF-2-neutralizing antibody for 6 hrs, and tube formation was observed under an inverted microscope. The images were captured with a CCD camera, and tube length was measured using the Micro Analyzer. **(a)** Control, **(b)** pravastatin (1 μ M), **(c)** FGF-2 antibody (5 μ g/ml), **(d)** FGF-2 antibody (5 μ g/ml) plus pravastatin (1 μ M), $N = 3$, * $P < 0.05$ versus (-)/(-).



Pravastatin-induced tube formation via FGF-2

Finally, we assessed the effect of FGF-2 antibody on pravastatin-induced tube formation. Pravastatin induced a 1.5-fold increase in tube formation. Treatment with neutralizing antibody did not influence basal tube formation in the absence of pravastatin but attenuated pravastatin-induced tube formation (Fig. 9C). Collectively, pravastatin-stimulated EC proliferation, migration and tube formation can be blocked by neutralizing FGF-2 activity.

Discussion

The novel finding in the present study is that ERK1,2 and Akt activation and subsequent EC proliferation in response to pravastatin is partially mediated by endogenous FGF-2 through the activation of the FGFR. We demonstrated that early activation of Akt by pravastatin is dependent upon FGF-2 and FGFR activation. Treatment with FGF-2 antibody or heparin inhibited the pravastatin-induced Akt activation, suggesting that FGF-2 is an autocrine initiation factor. Our data further suggest that endogenous FGF-2 contributes to MAPK activation by pravastatin because MAPK is affected by either FGF-2 blockade or FGFR inhibition. Because quenching FGF-2 by application of neutralizing antibodies

prevented endothelial proliferation, migration and tube formation, we conclude that pravastatin induces proangiogenic effects in an extracellular FGF-2-dependent manner.

FGF-2 is an angiogenic factor in the FGF superfamily [20]. The function of statins on angiogenesis is based on their influence on the expression of FGF-2. Donnini *et al.* have also reported that FGF-2 plays a central role in the prosurvival/proangiogenic effects of angiotensin-converting enzyme inhibitors (ACEI) [21]. Because the MAPK and PI3K/Akt pathways are known to contribute to FGF-2-initiated signalling [22], we investigated the effect of pravastatin on the FGFR. In particular, FGFR-1 has been shown to play an essential role in FGF-2-induced effects in ECs [23] Therefore, FGFR-1 is activated by pravastatin in a time-dependent manner. We could not detect pravastatin-induced phosphorylation of the VEGF receptor (data not shown). Furthermore, pravastatin had no effect on FGF-2 protein levels within 1 hr (data not shown), indicating that the up-regulation of FGF-2 is not necessary for pravastatin-induced early cellular response.

Heparan sulphate proteoglycans (HSPGs) function as an extracellular matrix or surface reservoir for FGF-2 [24, 25]. FGF-2 molecules are anchored to HSPGs with a lower affinity than to FGFRs, and are required cofactors for the activation of FGFR [25, 26]. Binding to HSPGs protects FGF-2 from degradation, whereas other unknown factors on the cell surface are required for FGF-2 signalling [25, 27]. Heparin displaces FGF-2 from the cell surface, thus preventing its binding to FGFR-1 [25, 26].

Pravastatin might mobilize FGF-2 from HSPGs, thereby enabling the free FGF-2 to bind and activate FGFR, because heparin-treatment inhibited pravastatin-induced MAPK and Akt activation. Previous reports have also suggested that FGF-binding protein (FGF-BP) and CYR61 act in part by displacing FGF-2 bound to the extracellular matrix, thus increasing the effective concentration of FGF-2 [28, 29]. These molecules may be associated with pravastatin-induced FGF-2/FGFR activation. Interestingly, Soltys *et al.* have shown that fatty acids can block Akt activation [30]. Therefore, one may speculate that statins somehow interact with lipid signalling. Lipid rafts are cholesterol-rich membrane domains in which FGF-2-binding HSPGs, like syndecan-4, cluster. Whether the distribution of lipid rafts contributes to the pravastatin-induced pleiotropic effects of FGF-2 or is involved in the binding of FGF-2 on FGFR is unknown. Thus, pravastatin acts as a primer of the angiogenic switch by promoting FGF-2/FGFR interaction through the mobilization of FGF-2 from the extracellular matrix.

It would be interesting to know whether the FGF-2/FGFR1 pathway is common for lipophilic statins. To evaluate the contribution of the FGF-2/FGFR pathway to the angiogenic effects of statins, we investigated the effects of the lipophilic statins simvastatin and pitavastatin on the activation of Akt and ERK1,2 (Fig. 8). As a result, we found that the FGF-2-neutralizing antibody inhibited both simvastatin and pitavastatin-induced ERK1,2 activity. Interestingly, FGF-2-neutralizing antibody partially suppressed pitavastatin-induced Akt activation. However, simvastatin-induced Akt activation was not inhibited. These data suggest that the effects of lipophilic statins on the activation of Akt and ERK1,2 were mediated, at least in part, by the participation of extracellular FGF-2. Activation of the MAPK pathway induced by lipophilic statins might be dependent on the extracellular FGF-2 level. In contrast, the activation of the PI3K/Akt pathway, as previously reported, may be due to the statins that permeate through the cell membrane. From these findings, we hypothesize that all statins might increase the fluidity of extracellular FGF-2. However, we conclude that the dependence on the FGF-2/FGFR pathway of

statins is not entirely common to all statins. Further evaluation is required to elucidate whether other statins contribute to the FGF-2/FGFR pathway.

The existing literature regarding the pleiotropic effect of statins on angiogenesis has been puzzling. Statin has a biphasic and dose-dependent effect on angiogenesis. Statins play a dual role in angiogenesis signalling by promoting cell migration at low concentrations and exerting antiangiogenic effects at high concentrations. We have reported that low concentrations of pravastatin (100 nM and 1 μ M) enhanced rAEC proliferation, whereas higher concentrations of the statin (10 μ M and 100 μ M) did not [17]. These results may be attributed to the dependence on the activation of the FGF-2/FGFR pathway.

In summary, the participation of extracellular FGF-2 is another potential mechanism by which statins may promote angiogenesis. Our data indicate that FGF-2 is a trigger in pravastatin-induced proangiogenic effects. Thus, activation of extracellular FGF-2/FGFR-dependent Akt phosphorylation mediates, at least in part, the pleiotropic effects of statins on the endothelium.

Acknowledgements

The authors would like to thank Azusa Inagaki for technical assistance and the members of the Central Laboratory of Osaka City University Graduate School of Medicine, for providing technical support. This study was supported by a Grant-in-Aid for Young Scientists (B) (#19790389) to M.S. and Grant-in-Aid for Scientific Research (B) (#17390068) to H.I. from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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