Original Article

Fluvastatin Upregulates the Expression of Tissue Factor Pathway Inhibitor in Human Umbilical Vein Endothelial Cells

Akiko Sekiya[1], Eriko Morishita[1], Keiko Maruyama[2], Hiroki Torishima[1] and Shigeki Ohtake[1]

[1]Department of Clinical Laboratory Science, Kanazawa University Graduate School of Medical Science, Ishikawa, Japan
[2]National Cerebral and Cardiovascular Center, Department of Molecular Pathogenesis, Osaka, Japan

***Aim*: 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are cholesterol-lowering drugs with a variety of pleiotropic effects including antithrombotic properties. Tissue factor pathway inhibitor (TFPI), which is produced predominantly in endothelial cells and platelets, inhibits the initiating phase of clot formation. We investigated the effect of fluvastatin on TFPI expression in cultured endothelial cells.**

***Methods*: Human umbilical vein endothelial cells (HUVECs) were treated with fluvastatin (0–10 μM). The expression of TFPI mRNA and antigen were detected by RT-PCR and western blotting, respectively. The effects of mevalonate intermediates, small GTP-binding inhibitors, and signal transduction inhibitors were also evaluated to identify which pathway was involved. A luciferase reporter assay was performed to evaluate the effect of fluvastatin on TFPI transcription. The stability of TFPI mRNA was estimated by quantitating its levels after actinomycin D treatment.**

***Results*: Fluvastatin increased TFPI mRNA expression and antigen in HUVECs. Fluvastatin-induced TFPI expression was reversed by co-treatment with mevalonate or geranylgeranylpyrophosphate (GGPP). NSC23766 and Y-27632 had no effect on TFPI expression. SB203580, GF109203, and LY294002 reduced fluvastatin-induced TFPI upregulation. Moreover, fluvastatin did not significantly affect TFPI promoter activity. TFPI mRNA degradation in the presence of actinomycin D was delayed by fluvastatin treatment.**

***Conclusions*: Fluvastatin increases endothelial TFPI expression through inhibition of mevalonate-, GGPP-, and Cdc42-dependent signaling pathways, and activation of the p38 MAPK, PI3K, and PKC pathways. This study revealed unknown mechanisms of the anticoagulant effect of statins and gave a new insight to its therapeutic potential for the prevention of thrombotic diseases.**

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Key words: 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, Fluvastatin, Tissue factor pathway inhibitor, Human umbilical vein endothelial cells

Introduction

Activation of the extrinsic coagulation pathway triggers arterial thrombotic events, such as acute coronary syndrome, ischemic stroke, and critical limb ischemia. Thrombin formation is further accelerated at the site of atherosclerotic plaques, where a chronic inflammatory response occurs and collagen and tissue factor (TF) are exposed to circulating blood after the rupture of an atherosclerotic plaque. Tissue factor pathway inhibitor (TFPI), as the major inhibitor of the extrinsic coagulation pathway, regulates arterial thrombosis by binding TF-factor VIIa (TF-FVIIa) and factor Xa (FXa). Recent studies have shown that TFPI attenuates the development of atherosclerosis by

Address for correspondence: Eriko Morishita, Department of Clinical Laboratory Science, Kanazawa University Graduate School, 5-11-80 Kodatsuno, Kanazawa, Ishikawa 920-0942, Japan
E-Mail: eriko86@staff.kanazawa-u.ac.jp
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inhibiting endothelial activation and proliferation, and by diminishing monocyte recruitment[1]. Alternative splicing at the 3′ end of the TFPI gene results in the production of two major isoforms of TFPI, TFPIα and TFPIβ, which have different domain structures and tissue distributions. TFPIα circulates in the plasma or bound to the endothelium via its interaction with endothelial glycosaminoglycan. Another pool of TFPIα is released from endothelial cells and platelets after stimulation (i.e., thrombin). Thus, TFPIα acts as a very early phase inhibitor at the locus of clot formation. TFPIβ binds directly to the endothelium surface and contributes to constant anticoagulation on the vascular endothelium[2, 3].

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have been suggested to reduce the risk of cardiovascular events and death. Besides their predominant effects on cholesterol reduction, statins have shown a number of beneficial effects including the improvement of endothelial function, suppression of inflammation, and plaque stabilization[4]. These "pleiotropic" effects are independent of its effect on cholesterol reduction and mainly through inhibition of mevalonic acid synthesis, leading to the decreased synthesis of isoprenoids. Isoprenoids, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), mediate the posttranslational modification of small GTP-binding proteins of the Ras/Rho family[4]. Recent studies have shown the anticoagulant properties of statins[5]. The downregulation of TF and plasminogen activator inhibitor type 1 and the upregulation of thrombomodulin through inhibition of small GTP-binding proteins by statins were demonstrated *in vitro*[6-8]. Meanwhile, it remains unclear if statins affect TFPI expression. Although some reports, including ours, described the association of statin administration and plasma TFPI concentration[9-11], plasma TFPI does not reflect the amount of TFPI pooled in platelets and on the endothelium. Thus, we considered it beneficial to clarify if statins affect endogenous TFPI production *in vitro*.

In this report, we examined the effects of fluvastatin, a lipid-soluble statin, on TFPI expression in human endothelial cells and investigated its underlying mechanisms.

Methods

Materials

Human umbilical vein endothelial cells (HUVECs) and conditioned medium (EGM-2 Bullet Kit) were purchased from Lonza (Walkersville, MD, USA). EGM-2 was added with all of the attached supplements and 2% fetal bovine serum (FBS). Fluvastatin, mevalonate, FPP, and GGPP were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). FPP and GGPP were dissolved in methanol/10 mmol/L NH_4OH (vol/vol, 7/3). Y-27632 and NSC23766 were purchased from Calbiochem (San Diego, CA, USA). SB203580, U0126, SP600125, LY294002, and GF109203 were purchased from Alexis Biochemicals, Inc. (San Diego, CA, USA) and dissolved in dimethyl sulfoxide (DMSO). Actinomycin D was purchased from Sigma Aldrich Co. and dissolved in DMSO. An anti-TFPI polyclonal antibody was purchased from Haematologic Technologies, Inc. (Essex Junction, VT, USA). Polyclonal antibodies against p38 mitogen-activated protein kinases (MAPK), phospho-p38 MAPK, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Cell Culture

HUVECs (3–8 passages) were grown to confluence in EGM-2 at 37℃ in a humidified atmosphere of 5% CO_2. A human umbilical cell line (EA.hy926) was grown in Dulbecco's modified Eagle's medium containing 10% FBS at 37℃ in a humidified atmosphere of 5% CO_2.

Quantification of mRNA

Total RNA was extracted from cultured cells by using a NucleoSpin kit (NIPPON Genetics, Inc., Tokyo, Japan). The cDNA was synthesized by reverse transcription with a PrimeScript RT-PCR Kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. The cDNA was subjected to the following PCR conditions to amplify TFPI mRNA: 30 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 30 s. The following primers were used: TFPI forward 5′-TGGATGCCTGGGCAATATGA-3′ and reverse 5′-TATTCCAGCATTGAGCTGGGTTC-3′; and GAPDH forward 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse 5′-ATGGTGGTGAAGACGCCAGT-3′. PCR products were subjected to electrophoresis in a 3% agarose gel and the intensity of the bands was measured using a Typhoon9200 imager (GE Healthcare, Buckinghamshire, UK). The band intensity of the TFPI PCR products was normalized to that of GAPDH in the same samples.

Western Blot

HUVECs were lysed in a buffer comprising 50 mM Tris-HCl, pH 7.5, 1% bovine serum albumin, 2 mM EDTA, 100 U/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 200 mmol/L phenylmethanesulfonyl fluoride. HUVEC lysates were electrophoresed in a 10% SDS/polyacrylamide gel and transferred to a nitrocellulose membrane (Hypond™-P; GE Healthcare). Immunoblotting was performed using primary antibodies against TFPI, followed by secondary antibodies conjugated with horseradish peroxidase. The protein bands were visualized with Immobilon Western HRP Detection Substrate (Merck Millipore, Darmstadt, Germany).

Plasmid Construction for the Luciferase Reporter Assay

According to the TFPI promoter sequence reported by Petit *et al.*, a 1,524 bp (-1,246 and +278) DNA fragment of the 5′-flanking region of the TFPI gene was PCR-amplified using human genomic DNA as a template and the following specific primers containing restriction sites[12]: 5′-GGCTGCTAGCTTTGATTGTG-3′ (containing an *Nhe*I restriction site) and 5′-GCCAGGTACTCACAAGTAAGATCT-3′ (containing a *Bgl*II restriction site). This fragment was digested at the restriction sites and cloned between the unique corresponding sites of the pGL3 basic vector (Promega, Madison, WI, USA).

DNA Transfection and Luciferase Assays

EA.hy926 cells (2.0×10^5 cells/well) were allowed to grow on 12-well plates until they were approximately 80% confluent. The cells were then transiently co-transfected with 0.5 μg TFPI promoter constructs and 0.1 μg internal control vector pRL-TK (Promega) by using the Lipofectin reagent (Invitrogen, Carlsbad, CA, USA). After incubation for 6 h, the medium was replaced by fresh medium and the cells were allowed to grow for another 20 h prior to fluvastatin treatment. Luciferase activity in the cell lysates was determined using the dual-luciferase reporter assay system (Promega) and a luminometer (Atto, Tokyo, Japan) after treatment of the cells with fluvastatin (0–10 μM) for 24 or 48 h.

MTT Assay

The cells were seeded into the wells of 96-well microplates at a density of 5,000 cells/well. After incubation in the designated condition, the medium was replaced with 100 μL fresh culture medium. Then, 10 μL MTT stock solution (5 mg/mL in sterile phosphate-buffered saline) were added to each well. After 3 h, the unreacted dye was removed, and the formazan crystals were dissolved in 150 μL DMSO. After gentle agitation for 5 min, absorbance was read at 570 nm using a Microplate Reader (Tecan, Kanagawa, Japan). Cells incubated in medium without supplementation were considered as controls, and the viability of the control cells was set to 100%.

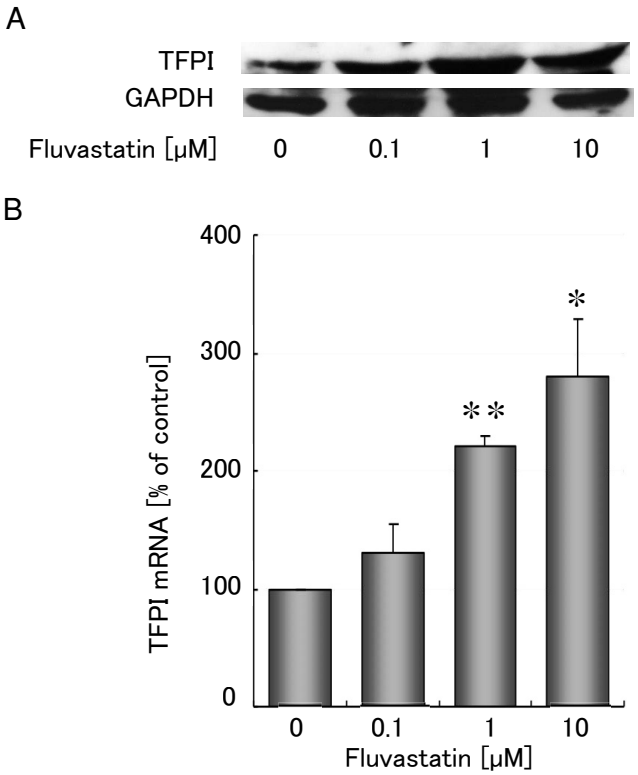

Fig. 1. Fluvastatin upregulates the TFPI antigen and mRNA expression in HUVECs.

HUVECs were incubated with various concentrations (0–10 μM) of fluvastatin for 24 h. mRNA and protein levels were determined by RT-PCR (B) and western blotting (A), respectively. The data represent the mean ± SD of 3 separate experiments. $^*p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control.

Statistical Analysis

Each experiment was performed in triplicate and the results are expressed as the mean ± standard deviation (SD). Student's *t* test and one-way analysis of variance were used for statistical analyses. Microsoft Excel 2007 was applied for all analyses. A value of $p < 0.05$ was considered statistically significant.

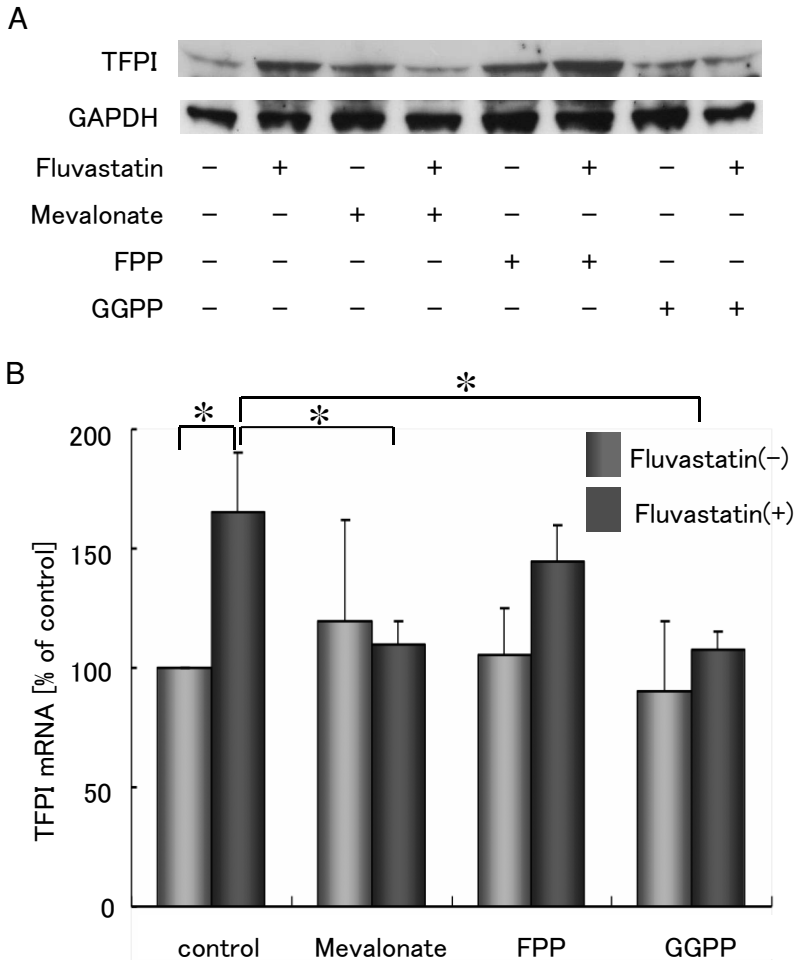

Fig. 2. Fluvastatin-induced upregulation is reversed by mevalonate and GGPP, but not FPP.

HUVECs were incubated with mevalonate (200 μM), FPP (10 μM), or GGPP (10 μM) in the presence or absence of fluvastatin (1 μM) for 24 h. mRNA and protein levels were determined by RT-PCR (B) and western blotting (A), respectively. The data represent the mean ± SD of 3 separate experiments. $^*p < 0.05$ vs. control.

Results

Fluvastatin Upregulates TFPI Antigen and mRNA Expression in HUVECs

Treatment of HUVECs with fluvastatin (0.1–10 μM) for 24 h significantly increased TFPI antigen levels in a concentration-dependent manner (**Fig. 1A**). The increase of TFPI antigen levels was accompanied by the increased expression of TFPI mRNA, which showed a 2-fold increase compared to untreated cells after treatment with 1 μM fluvastatin for 24 h ($p < 0.01$, **Fig. 1B**). Treatment of HUVECs with 10 μM fluvastatin for 24 h did not affect their viability (data not shown).

Fluvastatin Upregulates TFPI Expression Through Inhibition of the Mevalonate Pathway

To clarify further whether the induction of TFPI expression by fluvastatin was mediated by inhibition of the mevalonate pathway, we incubated HUVECs with mevalonate (200 μM), FPP (10 μM), or GGPP (10 μM) in the presence or absence of fluvastatin (1 μM) for 24 h. As shown in **Fig. 2**, treatment of HUVECs with mevalonate and GGPP reversed the induction of TFPI antigen (**Fig. 2A**) and mRNA (165 ± 25 vs. 110 ± 10%, $p < 0.05$ and 165 ± 25 vs. 108 ± 7%, $p < 0.05$, respectively, **Fig. 2B**). In contrast, FPP did not significantly alter the effect of fluvastatin on TFPI induction (165 ± 25 vs. 145 ± 15%, **Fig. 2B**).

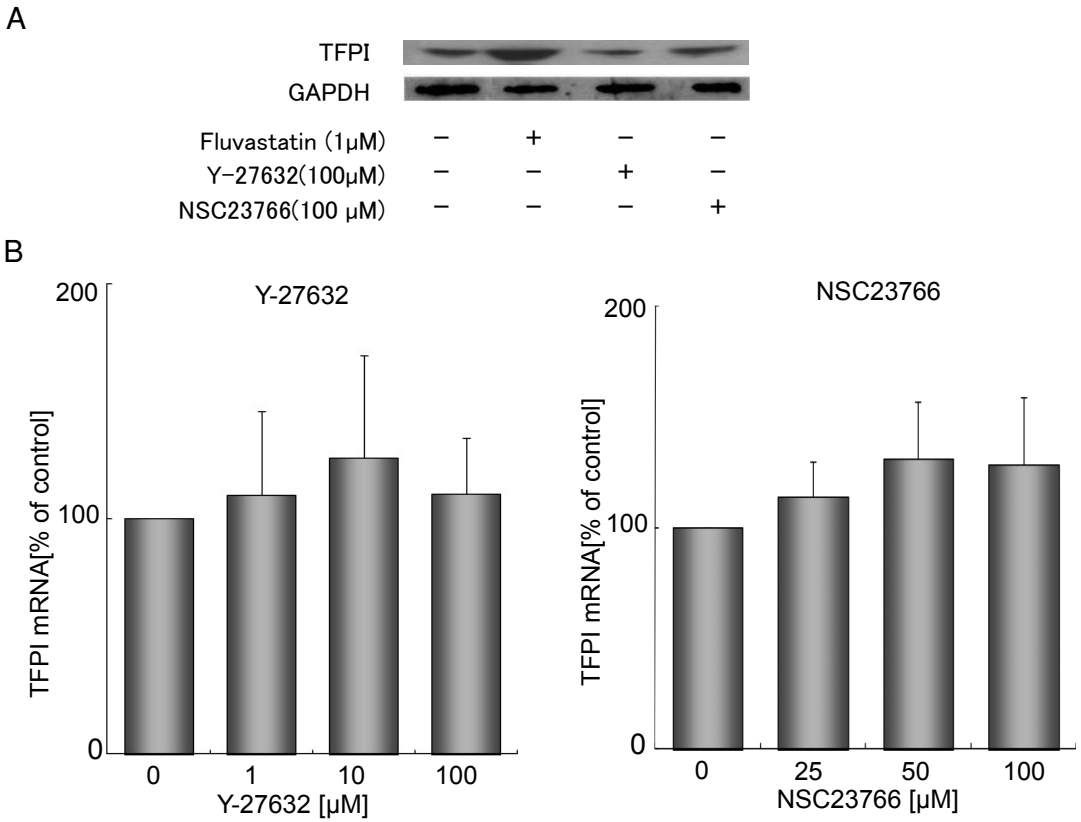

Fig. 3. Y-27632 and NSC23766 have no effect on the fluvastatin-induced upregulation of the TFPI expression in HUVECs.

HUVECs were incubated with Y-27632 (1–100 μM) or NSC23766 (25–100 μM) for 24 h. After incubation, mRNA and protein levels were determined by RT-PCR (B) and western blotting (A), respectively. The data represent the mean ± SD of 3 separate experiments.

Rho and Rac Inhibition are not Involved in Fluvastatin-Induced TFPI Expression

Since isoprenylation of the small GTP-binding proteins Rho and Rac is mediated by GGPP, we tested whether inhibition of these small GTP-binding proteins is involved in TFPI induction. Treatment of HUVECs with NSC23766 (25–100 μM) and Y-27632 (1–100 μM), specific inhibitors of Rac- and Rho-dependent kinases, respectively, showed no significant effect on TFPI protein (**Fig. 3A**) and mRNA (**Fig. 3B**) expression.

p38 MAPK, PKC, and PI3K Pathways are Involved in Fluvastatin-Induced TFPI Expression

We then examined the involvement of signal transduction pathways in the upregulation of TFPI expression by fluvastatin. Treatment of HUVECs with SB203580 (p38 MAPK inhibitor, 20 μM), LY294002 (PI3K inhibitor, 10 and 20 μM), and GF109203 (PKC inhibitor, 0.1 and 1 μM) reduced fluvastatin-induced TFPI expression (**Fig. 4A** and **B**). In contrast, U0126 (ERK1/2 inhibitor, 20 μM) and SP600125 (JNK inhibitor, 20 μM) did not have a significant effect (**Fig. 4A**). In addition, increased phosphorylation of p38 MAPK was detected after fluvastatin treatment of HUVECs (**Fig. 4C**).

Fluvastatin Upregulates TFPI Expression without Enhancing its Promoter Activity Through mRNA Stabilization

To confirm the effects of fluvastatin on TFPI gene transcription, we transfected EA.hy926 cells with the TFPI promoter construct. Luciferase assays did not show a significant increase of TFPI promoter activity after treatment of the cells with fluvastatin (0–10 μM) for 24 or 48 h (**Fig. 5A**). We also confirmed that treatment with fluvastatin for 48 h induced TFPI mRNA expression in EA.hy926 cells (1 μM: 1.5-fold, 10 μM: 2.0-fold, data not shown).

We next investigated whether fluvastatin-induced

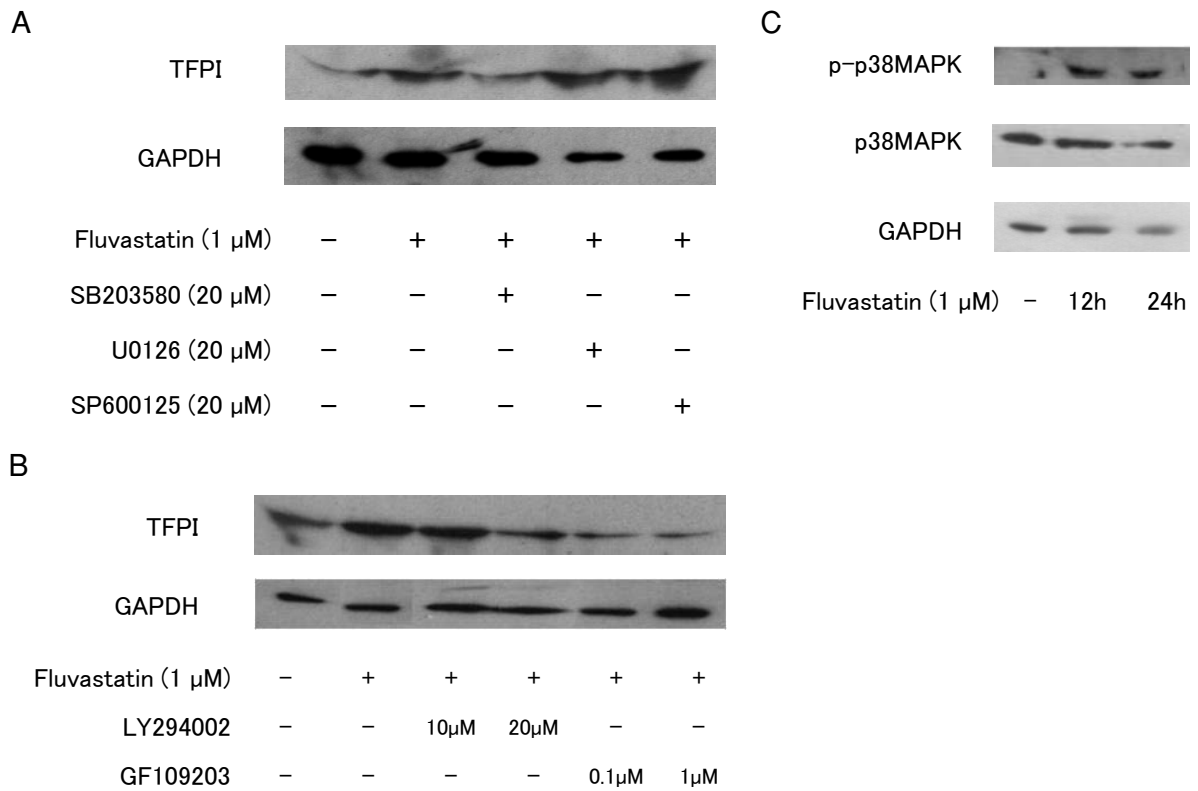

Fig. 4. Fluvastatin-induced TFPI expression is mediated via the activation of p38 MAPK, PKC, and PI3K in HUVECs.

A, HUVECs were incubated with fluvastatin (1 μM) in the presence or absence of SB203580 (p38 MAPK inhibitor, 20 μM), U0126 (ERK1/2 inhibitor, 20 μM), or SP600125 (JNK inhibitor, 20 μM) for 24 h; and B, GF109203 (PKC inhibitor, 0.1, 1 μM) or LY294002 (PI3K inhibitor, 10, 20 μM) for 24 h. After incubation, protein levels were determined by western blotting. C, HUVECs were incubated with fluvastatin (1 μM) for 12 or 24 h. Total or phosphorylated p38 MAPK was determined by western blotting.

TFPI expression was regulated via post-transcriptional mechanisms. The stability of TFPI mRNA was estimated by quantitating TFPI mRNA at various time points after the administration of a transcriptional inhibitor, actinomycin D (AD; 1 μg/mL), in the presence or absence of fluvastatin (1 μM). The amount of TFPI mRNA decreased gradually from 4 h after AD administration. In contrast, the amount of TFPI mRNA in the presence of fluvastatin remained steady (Fluvastatin + AD: 93 ± 9%, AD alone: 70 ± 6%, $p < 0.05$, **Fig. 5B**).

Discussion

Fluvastatin is reported to penetrate into vascular walls more effectively than other statins[13]. Previous reports have described that among all statins currently available, only fluvastatin interferes with proliferation of arterial smooth muscle cells at therapeutic levels[13, 14]. Therefore, we selected fluvastatin for use in this study, in combination with cultured human endothelial cells. We adjusted the experimental concentration of fluvastatin to 0.1–10 μM in consideration of the maximum blood concentration currently used (0.5–1 μM). It was demonstrated that fluvastatin upregulates TFPI expression in HUVECs. Moreover, some of underlying mechanisms of TFPI induction were identified. This is the first evidence to show the direct contribution of statins to TFPI expression. Together with the previously described anticoagulant effect of statins, their effect on TFPI induction seems to be a prospective therapeutic application for thrombotic diseases.

Our data showed that the fluvastatin-induced upregulation of TFPI was mediated by inhibition of the mevalonate and GGPP pathways. Nevertheless, it was shown that downstream small GTP-binding proteins, Rho and Rac, were not involved in the regulation of TFPI expression. Thus, inhibiting the remaining small GTP-binding protein, Cdc42, may contribute to TFPI induction by statins. Furthermore, it was

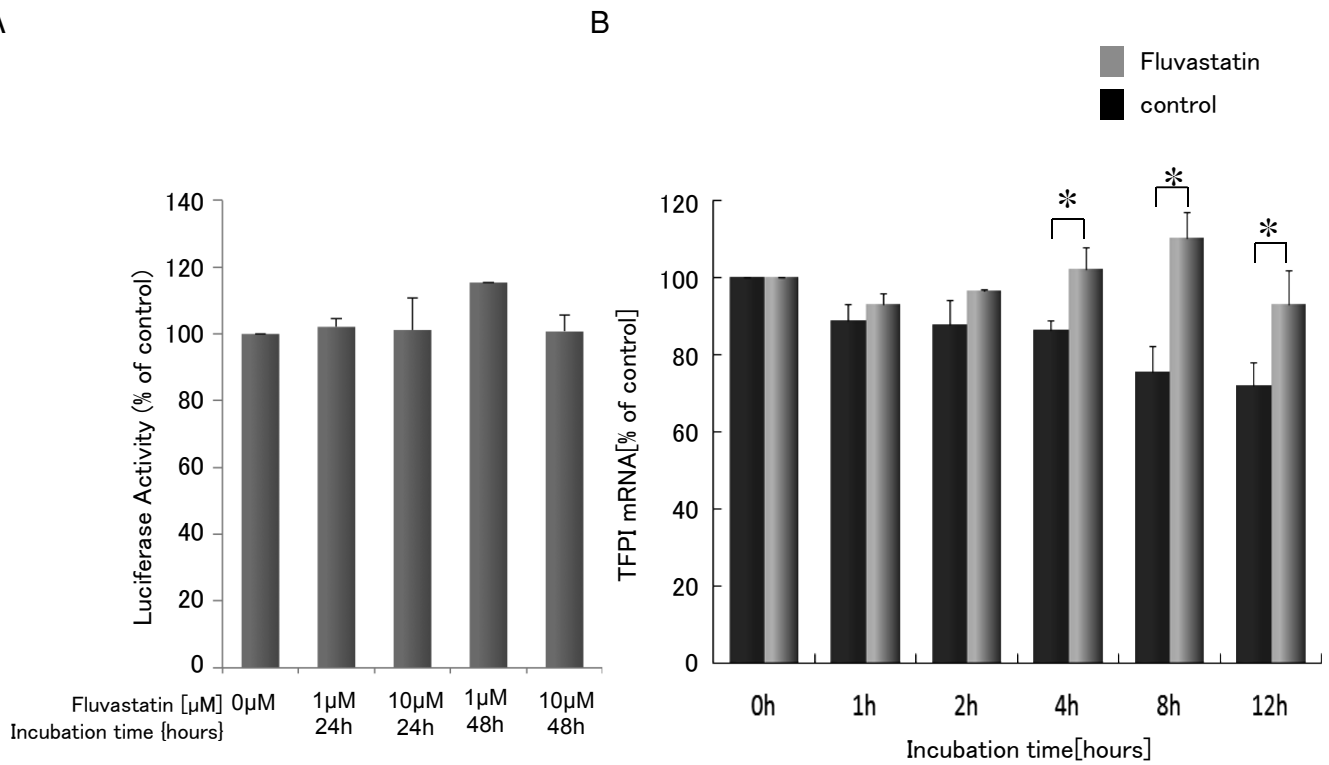

Fig. 5. Fluvastatin has no effect on the TFPI promoter activity in transiently transfected EA.hy926 cells and stabilizes TFPI mRNA in HUVECs.

A, EA.hy926 cells were transfected with a luciferase reporter gene construct containing the 5′-flanking region of the TFPI gene and were incubated with fluvastatin (0–10 μM) for 24 or 48 h. The data represent the mean ± SD of 5 separate experiments. B, HUVECs were incubated with fluvastatin (1 μM) for 24 h, and then actinomycin D (AD; 1 μg/mL) was added alone or with fluvastatin. The cells were harvested at the indicated time points and TFPI mRNA levels were determined by RT-PCR. The data represent the mean ± SD of 5 separate experiments. $^*p < 0.05$ vs. control.

suggested that activation of p38 MAPK, but not ERK or JNK, was involved in the upregulation of TFPI by fluvastatin. The statin-induced phosphorylation of p38 MAPK was demonstrated in some reports[15, 16]. Conversely, other reports described that statins showed their pleiotropic effects by attenuating the increased phosphorylation of p38 MAPK[17, 18]. The effect of statins on p38 MAPK phosphorylation may differ according to cell type and pretreatment condition of the cells. In our experiments, treatment of HUVECs with fluvastatin increased p38 phosphorylation; in addition, inhibition of p38 MAPK by SB203580 reversed the induction of TFPI by fluvastatin, suggesting that p38 MAPK activation by fluvastatin was involved in TFPI upregulation. The induction of TFPI was also reversed by the presence of LY294002 and GF109203, indicating that fluvastatin increased TFPI expression via the activation of PI3K and PKC. It is well documented that statins upregulate endothelial nitric oxide (NO) production via the PI3K/Akt signaling pathway[19]. The PI3K/Akt signaling pathway has various roles including cell metabolism, apoptosis, and proliferation. Of those, some are mediated by the induction of NO production, while others are independent of NO[20, 21]. We confirmed that fluvastatin-mediated TFPI upregulation was not reversed by L-NAME, indicating that TFPI upregulation through PI3K/Akt activation is independent of NO production (data not shown).

The transcriptional activity of the TFPI promoter cloned in front of the luciferase gene was not altered by fluvastatin treatment. As explained in the Materials and Methods, our construct consisted of exon 1 of the TFPI gene and its 5′-flanking region, which contains three GATA-2, one SP-1, and two c-Mic binding sites[22]. Our results showed that the upregulation of TFPI by fluvastatin is not mediated by those transcription factors. Instead, fluvastatin treatment of HUVECs delayed the degradation of TFPI mRNA in the presence of actinomycin D, indi-

cating that fluvastatin increased TFPI synthesis by improving its mRNA stability. Indeed, mRNA stabilization by statins has been reported for various genes other than TFPI. Habara *et al*. reported that pitavastatin increases iNOS gene expression through stabilization of its mRNA in rat hepatocytes. The presence of its 3′-untranslated region (UTR) containing AU-rich elements (ARE, AUUU[U]A), which are associated with ARE-binding proteins such as HuR, played a key role in this stabilization[23]. According to the ARE database (http://brp.kfshrc.edu.sa/ARED/), the full-length TFPIα mRNA contains 17 AREs within its 3′-UTR, which may contribute to the regulation of TFPI expression. In contrast to TFPIα, TFPIβ mRNA expression seems to be less dependent on AREs since it has only 2 AREs within its 3′-UTR. Besides, the expression of TFPIβ is reportedly affected by the alternative splicing of exon 2 rather than AREs[24]. Since all three signaling pathways that contributed to TFPI induction in our study, p38 MAPK, PI3K/Akt, and PKC, reportedly have a role in ARE-mediated mRNA stabilization[25], the contribution of each signaling pathway to TFPI induction is unclear and still needs to be investigated.

Our luciferase reporter assay does not reflect the consequence of alternative splicing of exon 2 nor mRNA stabilization through the 3′-UTR. It might be worth investigating if fluvastatin regulates TFPI expression by affecting alternative splicing of the 5′- or 3′-regions of the TFPI gene. As described, TFPI has two major isoforms with different tissue distribution. Since the primers that we used to quantitate TFPI mRNA can bind to both TFPIα and -β mRNA, it is uncertain which of these isoforms was upregulated, and this still needs to be investigated.

The favorable point of the use of TFPI as an anticoagulant, as compared with other anticoagulant drugs currently in use, is that it targets TF-FVIIa and FXa, thereby contributing to the inhibition of the very early phase of clot formation. In this way, TFPI has been expected to become a prospective therapeutic agent for anticoagulation and endothelial protection. Nevertheless, several clinical trials of recombinant TFPI administration did not achieve favorable results[26, 27]. It is tempting to speculate that inducing endogenous TFPI in the endothelium and platelets by statins may inhibit clot formation more effectively than administering exogenous TFPI, as endothelial- and platelet-derived TFPI may have a role as an on-demand and locus-specific clotting inhibitor during the rupture of an atherosclerotic plaque. Previously reported downregulation of TF by statins may contribute synergistically with the induction of TFPI to prevent intravascular coagulation. Further studies including animal experiments are needed.

Conclusion

We demonstrated that fluvastatin increases TFPI expression in HUVECs by inhibiting the GGPP- and Cdc42-dependent signaling pathways and the activation of the p38 MAPK, PI3K, and PKC pathways. This study revealed unknown mechanisms underlying the anticoagulant effects of statins and gave a new insight to its therapeutic potential for the prevention of thrombotic diseases.

Conflicts of Interest

There are no conflicts of interest associated with this manuscript.

Acknowledgements

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