difference in the ADAMTS13 activity levels of patients having CTD with and without thrombosis. The present study implicated that there was no direct relation between ADAMTS13 activity and thrombosis.

Except SLE, SSC is an only disease subgroup that developed several THE cases. Diffuse type SSc has generalized involvement with inflammatory reactions but not limited type SSc. Then, we investigated the ADAMTS13 activity, VWF, and VWFpp levels in the limited- and diffuse-type SSC. However, there were no significant differences in the ADAMTS13 activity, VWF, and VWFpp levels of patients with limited-type and diffuse-type SSc. A further study with a larger population focusing the specific antibodies and thrombosis is required.

Patients having CTD with elevated VWF or VWFpp or a reduced ADAMTS13 activity might thus have a high risk for TTP or APS. Primary APS had a low frequency of TTP, however patients having CTD with APS might have a high risk for TTP. Reduced ADAMTS13 activity and elevated VWF or VWFpp were frequently observed in patients with RA and MCTD, suggesting that RA or MCTD might lead to a high risk for TTP.

In conclusion, decreased ADAMTS13 levels and elevated VWF and VWFpp levels were observed in patients with CTD. These abnormalities may have close relation to thrombosis and vascular injury in CTD.

Declaration of Conflicting Interests

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ORIGINAL ARTICLE

Genetic variations in complement factors in patients with congenital thrombotic thrombocytopenic purpura with renal insufficiency

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Abstract The congenital form of thrombotic thrombocytopenic purpura (TTP) is caused by genetic mutations in ADAMTS13. Some, but not all, congenital TTP patients manifest renal insufficiency in addition to microangiopathic hemolysis and thrombocytopenia. We included 32 congenital TTP patients in the present study, which was designed to assess whether congenital TTP patients with renal insufficiency have predisposing mutations in complement regulatory genes, as found in many patients with atypical hemolytic uremic syndrome (aHUS). In 13 patients with severe renal insufficiency, six candidate complement or complement regulatory genes were sequenced and 11 missense mutations were identified. One of these missense mutations, C3:p.K155Q mutation, is a rare mutation located in the macroglobulin-like 2 domain of C3, where other mutations predisposing for aHUS cluster. Several of the common missense mutations identified in our study have been reported to increase disease-risk for aHUS, but were not more common in patients with as compared to those without renal insufficiency. Taken together, our results show that the majority of the congenital TTP patients with renal insufficiency studied do not carry rare genetic mutations in complement or complement regulatory genes.

Keywords Atypical hemolytic uremic syndrome · Complement factors · Renal insufficiency · Thrombotic thrombocytopenic purpura · Upshaw–Schulman syndrome

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Introduction

Thrombotic thrombocytopenic purpura (TTP), one form of thrombotic microangiopathy, is characterized by microangiopathic hemolytic anemia and thrombocytopenia. The congenital form of TTP, also known as Upshaw–Schulman syndrome, is caused by a severe constitutional deficiency of ADAMTS13 due to homozygous or compound heterozygous *ADAMTS13* mutations [1, 2] and is assumed to represent less than 5 % of all TTP cases. The age at disease onset is variable. Some congenital TTP patients present with overt TTP soon after birth, others experience first signs only in adulthood, e.g., triggered by pregnancy, or even remain asymptomatic into their fifth or sixth decades of life [3, 4]. Clinical manifestations in congenital TTP are also heterogeneous. Besides the classical hematological

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findings, some patients show neurological symptoms, others kidney involvement up to renal failure. Both onset during pregnancy and kidney involvement are noteworthy as they are features also observed in atypical hemolytic uremic syndrome (aHUS), another type of thrombotic microangiopathy [5, 6]. Approximately 60 % of aHUS cases can be explained by dysregulation and/or excessive activation of the alternative pathway of the complement system due to mutations in complement regulatory genes (*CFH*, *MCP*, *CFI*, *THBD*), hyperfunctional mutations of complement factors (*C3*, *CFB*) or autoantibodies against complement factor H [5, 6]. Recently, mutations in the diacylglycerol kinase epsilon gene were reported to co-segregate with phenotypic aHUS [7, 8].

To explain the variable clinical phenotypes in congenital TTP, genetic mutations responsible for increased activation of the complement system may influence the severity of renal involvement and thus serve as disease modifiers. Of note in this context is the study of Noris et al. who reported on two sisters with congenital TTP who showed different clinical phenotypes [9]. One sister manifested exclusively with neurologic symptoms while the other sister had very severe renal insufficiency that required chronic dialysis. In the latter, a missense mutation in complement factor H was identified which was not present in the sister with neurologic symptoms only. These data suggest that increased activation of the complement system due to genetic mutations modifies the severity of renal involvement in congenital TTP, a scenario derived from a single-family and to be verified in a larger number of patients.

In the present study, we explored whether genetic mutations in complement or complement regulatory genes leading to increased activation of the complement system contribute to the clinical phenotype of congenital TTP patients with predominant renal involvement.

Patients and methods

Patients

From two congenital TTP cohorts, that of the Hemostasis Research Laboratory in Bern, Switzerland [10, 11] and the Japanese congenital TTP study [4], confirmed congenital TTP patients were selected based on the following two criteria: (1) the patient had not been extensively studied by other groups and (2) whole blood for DNA extraction was available. A total of 32 congenital TTP patients (30 from Europe and 2 from Japan) were included in this study, of which thirteen had severe renal insufficiency up to end-stage renal disease (Table 1). The definition for renal involvement was as follows: (1) acute renal insufficiency during one or more acute TTP bouts requiring dialysis, or (2) chronic kidney disease defined according to KDIGO (kidney disease: improving global outcomes) as persistence of a glomerular filtration rate (GFR) <60 ml/min/1.73 m^2 or albuminuria for at least 3 months, with or without arterial hypertension; or (3) end-stage renal disease requiring renal replacement therapy (either dialysis or kidney transplant); or (4) documented tissue damage on renal biopsy; or (5) having a diagnosis of (atypical) hemolytic uremic syndrome established by a nephrologist based on the concomitant presence of thrombocytopenia, microangiopathic hemolytic anemia and renal insufficiency. All patients had severe ADAMTS13 deficiency (<10 % of the normal) in the absence of a functional inhibitor on at least two time points, at least two ADAMTS13 mutations and/or a plasma infusion trial demonstrating full recovery of infused ADAMTS13 and a plasma half-life of ADAMTS13 of 2–4 days. The plasma ADAMTS13 activity was measured as previously described [12–14]. The study was approved by the Institutional Review Board of each institution.

Genetic analysis

The coding exons and flanking intronic regions of *CFH, C3, MCP, CFI, CFB,* and *THBD* were sequenced as described previously [15, 16] in all 13 congenital TTP patients presenting with renal insufficiency. In the remaining 19 European congenital TTP patients without renal involvement, only a limited analysis of the 11 genetic missense mutations identified in the patients with renal insufficiency was performed. The allele frequencies of the found 11 missense mutations between the 11 European congenital TTP patients with renal involvement and the 19 European congenital TTP patients without renal involvement were compared by Chi square analysis. The nomenclature system of the amino acid and nucleotide numbers is given according to the recommendation of the Human Genome Variation Society. The A of the ATG of the initial Met codon is denoted as nucleotide +1, and the initial Met residue is denoted as amino acid +1. Multiplex ligation-dependent probe amplification analysis was used to screen for deletions of *CFH* and *CFHR*s using a commercially available kit (MLPA kit P236-A2; MRC-Holland, the Netherlands) [15]. The possible impact of the identified genetic mutations on structure and function of the respective proteins was examined by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html). The crystal structure of the complex of C3b and CCP 1-4 domains of CFH (ID: 2WII) [17] was retrieved from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). Molecular graphic imaging and analysis were generated using the PyMOL molecular visualization system (Schrödinger, Portland, OR).

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Table 1 Clinical characteristics and genetic variations of 13 congenital TTP patients with renal insufficiency

Patient ID	Current age (y)	Sex	CFH			C3			CFI
			rs800292	rs1061170	rs1065489	rs2230199	rs147859257	rs1047286	rs145769028
			c.184G > A	c.1204T > C[a]	c.2808G > T	c.304C > G	c.463A > C	c.941C > T	c.603A > C
1	36	F		p.Y402H		p.R102G	**p.K155Q**[b]	p.P314L	
2	53	M		p.Y402H	p.E936D				
3	61	M	p.V62I						
4	33	M	p.V62I (homo)						
5	61	F		p.Y402H	p.E936D				
6	38	M	p.V62I			p.R102G		p.P314L	
7	29	F	p.V62I (homo)			p.R102G		p.P314L	
8	48	F	p.V62I	p.Y402H					
9	44	F		p.Y402H (homo)		p.R102G		p.P314L	
10	46	M							
11	29	F		p.Y402H	p.E936D	p.R102G		p.P314L	
12	43	M			p.E936D (homo)				
13	35	M	p.V62I		p.E936D (homo)				**p.R201S**[c]
NHLBI GO Exome Sequencing Project									
MAF			39 %	38 %	14 %	15 %	0.3 %	15 %	None
1000 Genomes Project									
MAF, EUR			26 %	36 %	18 %	22 %	0 %	21 %	0 %
MAF, JPT			41 %	7 %	46 %	0 %	0 %	0 %	2 %

Table 1 continued

Patient ID	CFB			THBD	Number of CFHR1-R3 allele	ADAMTS13			Clinical characteristics
	rs4151667 c.26T > A	rs641153 c.95G > A	rs4151651 c.754G > A	rs1042579 c.1418C > T		Amino acid and/or nucleotide change	Amino acid and/or nucleotide change	Activity (%)	
1	**p.L9H**[c]			p.A473V	2	p.D235H	p.W542G	3.5[d]	Adult onset during 1st pregnancy with renal complications and renal sequelae
2			**p.G252S**[c]		2	c.4143_4144 insA p.E1382Rfs*6	c.4143_4144 insA p.E1382Rfs*6	<1[d]	Acute bouts always with severe renal insufficiency
3			**p.G252S**[c]	p.A473V	0	c.4143_4144 insA p.E1382Rfs*6	c.4143_4144 insA p.E1382Rfs*6	<1[d]	Acute bouts with severe renal insufficiency requring dialysis; triggered by (mild/moderate) alcohol consumption
4		p.R32Q (homo)		p.A473V	2	c.4143_4144 insA p.E1382Rfs*6	c.4143_4144 insA p.E1382Rfs*6	<1[d]	Original diagnosis recurrent HUS
5				p.A473V	2	p.R507Q	c.4143_4144 insA p.E1382Rfs*6	<1[d]	End-stage renal disease and kidney transplantation
6		p.R32Q			1	p.G1239 V	p.G1239 V	<1[d]	In adulthood acute bouts always with severe renal insufficiency requring dialysis; triggered always by alcohol consumption
7					2	p.R692C	c.3044 + 2430_ 3568 + 81del3291	<3[e]	End-stage renal disease
8		p.R32Q			2	c.2000delA p.N667Tfs*31	p.R1219Q	3[d]	Renal sequelae
9					2	p.Y177C	p.R1060W	1.5 in acute bout, 5 in remission[d]	Original diagnosis aHUS/membranoproliferative glomerulonephritis
10		p.R32Q		p.A473V	0	p.L232Q	p.R1060W	1 in acute bout, 5.3 in remission[d]	Renal sequelae
11					2	p.R1095Q	not identified	<1[d]	Renal sequelae

Table 1 continued

Patient ID	CFB			THBD	Number of CFHR1-R3 allele	ADAMTS13			Clinical characteristics
	rs4151667 c.26T > A	rs641153 c.95G > A	rs4151651 c.754G > A	rs1042579 c.1418C > T		Amino acid and/or nucleotide change	Amino acid and/or nucleotide change	Activity (%)	
12				p.A473V	2	p.H234Q	p.R1206*	<0.5[f]	Acute bouts with renal insufficiency
13				p.A473V	2	c.1885delA p.R629Efs*69	p.C908Y	<0.5[f]	Original diagnosis aHUS; acute bouts with severe renal insufficiency requring dialysis; triggered by heavy alcohol consumption
NHLBI GO Exome Sequencing Project									
MAF	3 %	12 %	3 %	14 %					
1000 Genomes Project									
MAF, EUR	5 %	9 %	3 %	19 %					European (CEU, FIN, GBR, IBS, TSI), 1006 alleles
MAF, JPT	4 %	7 %	0 %	26 %					Japanese, 208 alleles

Patient ID 1–11 are from Swiss registry and patient ID 12 and 13 are from Japan registry. Nonsynonymous mutations were not identified in the MCP gene

The A of the ATG of the initial Met codon is denoted as nucleotide +1, and the initial Met residue is denoted as amino acid +1

The MAF of the NHLBI GO Exome Sequencing Project was obtained from http://evs.gs.washington.edu/EVS/, and the MAF of the 1000 Genomes Project Phase 3 was obtained from http://www.1000genomes.org/analysis

TTP thrombotic thrombocytopenic purpura, *aHUS* atypical hemolytic uremic syndrome, *MAF* minor allele frequency

[a] Reference sequence of CFH (NM 000186.3) is c.1204C > T

[b] Bold and underlined, rare and potentially predisposing mutation

[c] Bold, low frequency mutation

[d] The ADAMTS13 activity was measured by FRETS-VWF73 assay

[e] Quantitative immunoblotting assay, or

[f] Act-ELISA assay

In two European patients (Table 1, patient ID 7 and 11), only a single causative *ADAMTS13* mutation had been identified. Therefore, we employed the newly developed genomic quantitative PCR method [18] to identify a second causative mutation.

Results

To identify genetic mutations in complement genes leading to increased activation of the alternative pathway of the complement system, we performed DNA sequencing of the 6 candidate genes, *CFH*, *C3*, *MCP*, *CFI*, *CFB*, and *THBD* and identified 11 missense mutations in 13 congenital TTP patients with renal insufficiency (Table 1). We retrieved the allele frequency of these missense mutations from population cohorts participating in the NHLBI GO Exome Sequencing Project and the 1000 Genomes project Phase 3 and found that C3:p.K155Q is a rare mutation with a minor allele frequency (MAF) of 0.3 % and the two CFB missense mutations, p.L9H and p.G252S, are low frequency mutations with a MAF of 3 % (Table 1). These three missense mutations were observed in the European patients. One Japanese patient (ID 13) with renal insufficiency carried the one Japanese-specific CFI missense mutation, p.R201S, which is a low frequency mutation with a MAF of 2 % in the Japanese population [19]. The remaining 7 missense mutations were classified as common mutations with a MAF of more than 5 %.

We also genotyped the found 10 missense mutations, with exclusion of one Japanese-specific mutation, in the 19 European congenital TTP patients without renal involvement (data not shown). C3:p.K155Q was not identified in this group. None of the remaining 9 missense mutations found in the European congenital TTP patients was significantly more common among patients with compared to those without renal involvement.

Since the ADAMTS13 mutation, 4143_4144 insA, is frequent among patients with congenital ADAMTS13 deficiency in Northern and Central European countries [20], it was frequent in our cohort of European origin. Three of 11 (27.3 %) European congenital TTP patients with renal involvement were homozygous carriers for the frequent ADAMTS13 mutation 4143_4144insA, as were 6/19 (31.6 %) European congenital TTP patients without renal involvement. Though no difference in plasma ADAMTS13 activity between congenital TTP patients with and without renal involvement was observed, patients without renal involvement were younger (median 33 years, range 14–75 years) than patients with renal involvement (median 43 years, range 29–61 years).

We have recently developed a new genomic quantitative PCR method to identify large gene deletions in the *ADAMTS13* gene [18]. Employing this method, we identified a second causative mutation in patient ID 7 *ADAMTS13* c.3044 + 2430_3568 + 81del3291, a 3291-bp deletion including exons 24 and 25. In patient ID 11, no large deletion was observed and though the obligatory second mutation still remains unknown, the plasma infusion trial confirmed the congenital TTP diagnosis in this patient.

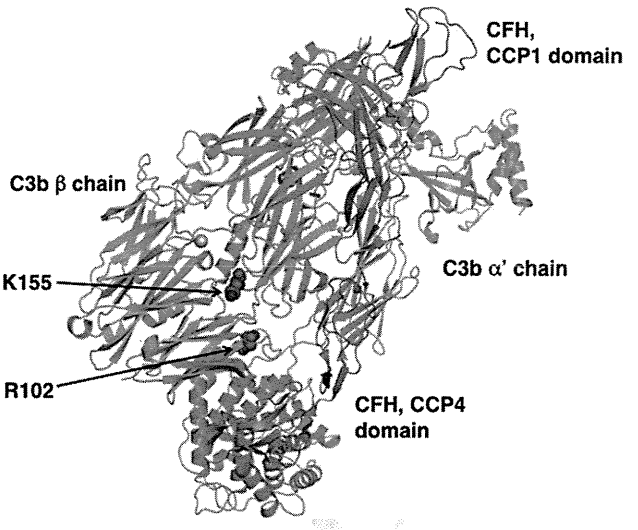

Fig. 1 Location of the C3:p.K155Q and C3:p.R102G mutations in the C3b-CFH CCP1-4 complex. C3b β-chain containing macroglobulin-like domains (MG) 1-5 is shown in *blue* and C3b α'-chain is shown in *green*. Complement control protein (CCP) domains 1-4 in CFH are depicted in *orange*. A calcium ion is shown as *gray sphere*. The p.K155 and p.R102 residues in C3b are depicted by *magenta spheres*. Both mutations, p.K155Q and p.R102G, are not positioned at the contact interface of the two proteins. Diagram was generated with the PyMOL molecular visualization system

Discussion

In the present study, we identified 11 missense mutations in six candidate complement (*C3*, *CFB*) and complement regulatory (*CFH*, *MCP*, *CFI*, *THBD*) genes in 13 congenital TTP patients with renal insufficiency and classified them into rare, low frequency, and common mutations.

The rare missense mutation, C3:p.K155Q found in patient ID 1 is located in the macroglobulin-like (MG) 2 domain of C3, where other mutations predisposing to increased complement activation were previously identified. Figure 1 depicts the location of the C3:p.K155 residue in the crystal structure of the complex of C3b and CFH CCP1-4 domains [17]. The p.K155Q mutation is positioned slightly away from the interface between C3b and CFH CCP1-4, making it unlikely that their interactions are directly affected. Prediction of the impact of this mutation by PolyPhen-2 and SIFT showed "benign" and "tolerated"

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effects, respectively. For a definite verdict, however, functional studies of the C3:p.K155Q mutation would be needed. In case of common mutation C3:p.R102G, which is also positioned away from the interface between C3b and CFH CCP1-4 (Fig. 1), experimental data indicate that this C3 variant weakly binds to CFH, resulting in reduced CFH cofactor activity thereby favoring alternative complement pathway amplification [21].

The low frequency mutation, CFB:p.L9H within the CFB signal peptide sequence has been reported to be protective for age-related macular degeneration [22]. The low frequency mutation CFB:p.G252S is located in the CFB linker region between the CCP3 domain and the von Willebrand factor A domain. Functional consequences of this mutation are unknown. On the basis of the CFI crystal structure, the low frequency mutation CFI:p.R201S resides on the surface region of the protein away from the proposed cofactor and/or substrate interaction sites, indicating a non-dysfunctional mutation [23]. This mutation is found only in Far East populations including Japanese [19].

The remaining seven mutations found in our cohort are commonly present in the general population. In the study of a rare renal affection, dense deposit disease, both p.R102G and p.P314L mutations in C3 were identified as genetic risk factors for developing this disease [24]. C3:p.R102G is also strongly associated with age-related macular degeneration with an estimated population attributable risk of 22 % [25, 26]. In our cohort, both C3:p.R102G and C3:p.P314L were in perfect linkage disequilibrium and five patients with renal insufficiency carried both mutations (Table 1), pointing to susceptibility for renal involvement through probable hyperactivation of the complement cascade.

Previous functional analyses indicated that the common mutations, CFH:p.V62, C3:p.G102, and CFB:p.R32, are disease-risk mutations [21, 27–29]. The combination of these three mutations yielded sixfold higher hemolytic activity compared to the protective mutations CFH:p.I62, C3:p.R102, and CFB:p.Q32 [21]. The patient with the rare C3:p.K155Q mutation (ID 1) and two other congenital TTP patients with renal involvement (ID 9 and 11) are carriers of the combined disease-risk mutations (Table 1). The CFH:p.E936D mutation in the CCP16 domain of CFH found in congenital TTP patients ID 2, ID 5, ID 11, ID 12 (in homozygous state) and ID 13 (in homozygous state) has been associated with aHUS [30, 31]. Although these common mutations are not extremely destructive, the combined effects of disease-risk mutations as well as the rare missense mutation C3:p.K155Q may influence susceptibility to renal involvement in congenital TTP patients with hereditary ADAMTS13 deficiency.

Two of 13 congenital TTP patients with renal insufficiency carried homozygous deletions of *CFHR1/CFHR3* genes (Table 1). The complete absence as well as barely detectable levels of CFHR1/CFHR3 are related to the occurrence of autoantibodies to CFH [32] that account for 5–10 % of aHUS cases [5]. Therefore, in addition to the above-mentioned missense mutations in complement genes, the deletion of *CFHR1/CFHR3* may contribute to renal affection in congenital TTP patients.

Taken together, our study demonstrates that most of the congenital TTP patients with renal insufficiency do not carry genetic mutations in complement or complement regulatory genes known to predispose to renal insufficiency. Second, although some congenital TTP patients with renal insufficiency were found to be carriers of common aHUS-risk mutations, such as CFH:p.V62, CFH:p.D936, C3:p.G102, CFB:p.R32, and homozygous deletions of *CFHR1/CFHR3* genes, these mutations were not more common than in the general population or in our 19 congenital TTP patients without renal involvement.

Microvascular platelet thrombosis and endothelial injury resulting from ADAMTS13 deficiency initiate the coagulation and fibrinolytic pathways, which in turn may contribute to complement activation in congenital TTP [33, 34]. Complement activation with consumption of complement factors has already been demonstrated during acute TTP episodes [34, 35]. Overactivation of the alternative complement pathway also leads to coagulation cascade activation. Our results suggest that rare predisposing complement genetic mutations do not contribute to a large extent to the phenotypic variability in congenital TTP patients. Further, the common aHUS-risk mutations in complement or complement regulatory genes observed in our small series of congenital TTP patients with renal insufficiency were equally frequent in congenital TTP patients without renal failure. Recruitment of larger number of congenital TTP patients with well-defined phenotypes will be necessary to obtain a full conclusion.

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Compliance with ethical standards

Conflict of interest Dr. Lämmle is a member of the Data Safety Monitoring committee of the BAX 930 Study testing rADAMTS13 in congenital TTP patients. Dr. Fujimura is a recipient of the research fund from Alexion Pharmaceuticals. Other authors have no conflict of interests.

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CASE REPORT

Fulminant bilateral cerebral infarction caused by paradoxical embolism in a patient with protein S Ala525Val substitution

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Key words
cerebral infarction, gene mutation, paradoxical embolism, protein S deficiency, thrombophilia.

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Abstract

We report a 42-year-old woman who developed sudden fulminant cerebral infarction in the bilateral middle cerebral artery territories, causing status epilepticus and a decreased level of consciousness. Investigation showed thrombus in the right soleus vein and a patent foramen ovale, but no obvious embolic source, such as atrial fibrillation or a carotid or cerebral artery atherosclerotic lesion. Blood coagulation tests showed decreased levels of free protein S (25%) and total protein S (52%), and decreased protein S activity (15%). The patient was diagnosed with cerebral infarction as a result of paradoxical embolism, and type I protein S deficiency. DNA sequencing identified a novel point mutation in the *PROS1* gene, leading to the amino acid substitution, Ala525Val. It should be noted that this protein S mutation can cause thrombophilia and cerebral infarction.

Introduction

Protein C (PC) and protein S (PS) are vitamin K-dependent anticoagulant proteins. Thrombin is the final protease in the blood coagulation cascade, and interacts with antithrombin to form the thrombin–antithrombin complex, which converts PC to activated PC (APC). APC inactivates the activated coagulation factors, Va and VIIIa.[1] This important negative feedback mechanism prevents excessive blood coagulation. PS is an indispensable cofactor for APC activity. Deficiency of PC or PS causes a hypercoagulable state leading to thromboembolism including deep venous thrombosis, pulmonary embolism and cerebral venous sinus thrombosis, and is usually an inherited autosomal dominant disorder.[2] Patients with a patent foramen ovale (PFO) have interatrial communication with the potential for right-to-left shunting, and are at risk of paradoxical embolism from deep venous thrombosis causing brain infarction. As PFO occurs in approximately 25% of otherwise healthy individuals, paradoxical embolism should be considered as a cause of brain infarction in patients with thrombophilia.[3] Inherited PC and PS deficiencies are more common in Japan than in Western countries. Many point mutations resulting in PC or PS deficiency have been reported to date.[1] We report a patient with fulminant cerebral infarction in the bilateral middle cerebral artery (MCA) territories, caused by paradoxical embolism. The patient had PS deficiency with a previously unreported gene mutation.

Case Report

A 42-year-old woman was transferred to the National Hospital Organization Fukuoka Higashi Medical Center, Fukuoka, Japan, in an ambulance with status epilepticus and a decreased level of consciousness. She had neither a past history of epilepsy and pregnancy loss nor a family history of thrombotic diseases, and was not taking any medications. She had slept in the passenger seat while her family traveled by car overnight, and when she got out of the car and started to walk, she fell down and immediately lost consciousness. On admission, her blood pressure was 126/64 mmHg, heart rate was 101 b.p.m. regular, temperature was 36.4°C and respiratory rate was 24 breaths/min. She had a decreased level of consciousness, conjugate deviation of her eyes to the right and tonic–clonic convulsions of all extremities. Laboratory data were almost normal except for a high D-dimer level of 14.7 μg/mL. Atrial blood gas analysis under oxygen administration on administration was normal, except for hyperoxygenation (pH 7.364, pO_2 520.5 mmHg, pCO_2 46 mmHg and HCO_3^- 25.6 mEq/L). Chest X-ray and contrast computed tomography showed no

signs of pulmonary embolism. Cerebrospinal fluid examination was within the normal limits, with a cell count of 3/μL, protein concentration of 29.4 mg/dL and glucose concentration of 61 mg/dL. Diffusion-weighted magnetic resonance imaging showed increased signal intensity in the bilateral MCA territories, indicating bilateral acute brain infarction (Fig. 1a). Magnetic resonance angiography showed stenosis of the left MCA (Fig. 1b), and magnetic resonance venography showed no abnormality (Fig. 1c). Transesophageal echocardiography showed passage of microbubbles from a peripheral vein through the right atrium to the left atrium, indicating a PFO (Fig. 1d). Contrast-enhanced computed tomography showed thrombus in the right soleus vein (Fig. 1e). Blood coagulation and fibrinolysis tests showed decreased levels of free PS (25%) and total PS (52%), and decreased PS activity (15%). DNA sequencing of the *PROS1* gene identified a previously unreported mutation of GCC to GTC, resulting in the amino acid substitution of Ala525 by Val (Ala525Val; Fig. 1f). The patient was diagnosed with cerebral infarction as a result of paradoxical embolism, and type I PS deficiency. Follow-up magnetic resonance angiography showed recanalization of the left MCA (Fig. 1g), supporting an embolic mechanism. Despite intensive medical therapy, the patient still had total aphasia and quadriplegia when she was transferred to another hospital after 59 days.

Discussion

We report a young woman with sudden fulminant bilateral cerebral infarction resulting in status epilepticus, a decreased level of consciousness, total aphasia and quadriplegia. Extensive investigation did not find an obvious embolic source, but found venous thrombosis in the right lower extremity and PFO. She also had PS deficiency with a previously unreported point mutation. Considering all these findings, she was diagnosed with cerebral infarction caused by paradoxical embolism.

As aforementioned, both PS deficiency and PC deficiency are relatively common causes of thrombophilia in Japan. PS

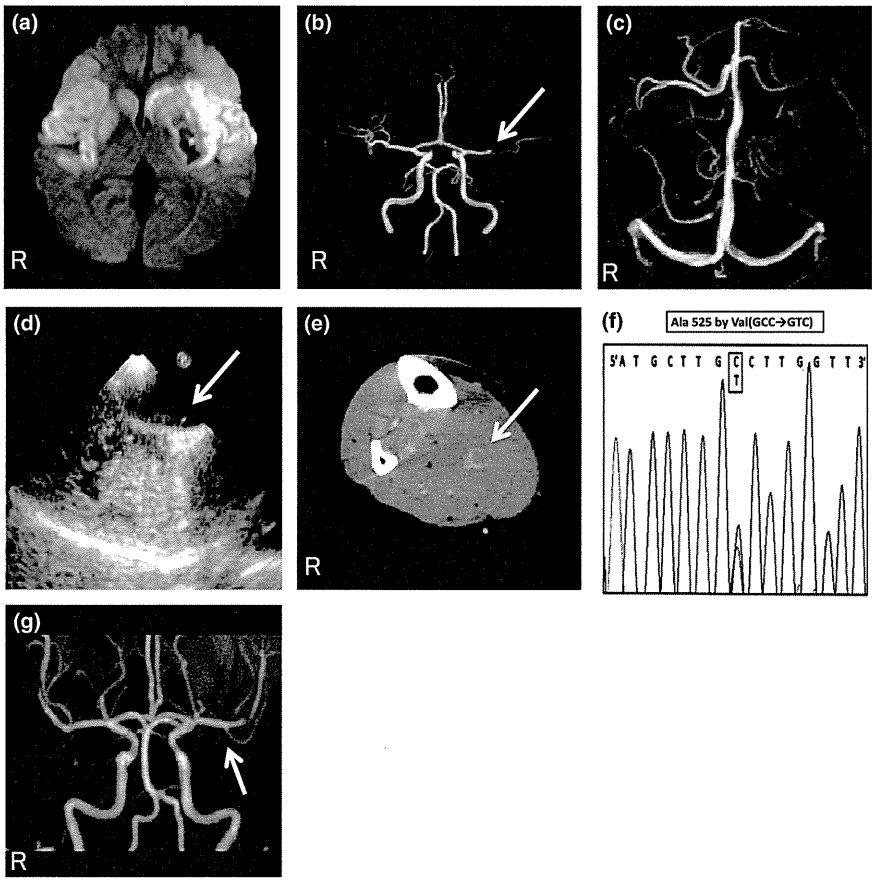

Figure 1 (a) Diffusion-weighted magnetic resonance imaging showed high-intensity lesions in the bilateral middle cerebral artery territories. (b) Magnetic resonance angiography showed stenosis of the left middle cerebral artery (arrow). (c) Magnetic resonance venography showed no abnormality. (d) Transesophageal echocardiography showed a patent foramen ovale (arrow). (e) Contrast-enhanced computed tomography showed thrombus in the right soleus vein (arrow). (f) Direct DNA sequencing identified the mutation GCC→GTC in the *PROS1* gene, resulting in the amino acid substitution of Ala-525 by Val. (g) Follow-up magnetic resonance angiography showed recanalization of the left middle cerebral artery.

circulates free in the plasma, or in a complex with complement protein C4b-binding protein. Only the free form acts as a cofactor to activate APC.[4] PS deficiency is classified into three types. Type I has decreased plasma levels of both free PS and total PS, and decreased PS activity; type II has normal free PS and total PS levels, and decreased PS activity; and type III has a normal total PS level, decreased free PS level and decreased PS activity.[5] Our patient was classified as type I deficiency, because she had decreased levels of free PS (25%) and total PS (52%), and decreased PS activity (15%).

The structure of PS includes an N-terminal Gla domain, thrombin-sensitive region, growth factor region and C-terminal sex-hormone binding globulin (SHBG)-like domain. Ala-525 is a well-conserved amino acid among different species that is located in the SHBG-like domain.[1] Substitution of Ala-525 by Pro, resulting from the point mutation of GCC to CCC, was previously reported to cause type I PS deficiency, similar to the present case.[6] As Ala-525 constitutes a β-strand, substitution of Ala with a more bulky amino acid, such as Val or Pro, might disrupt the structural stability of the protein, thereby decreasing the levels of both free PS and total PS.[1]

In conclusion, we experienced a case of paradoxical cerebral embolism in a patient with a previously unreported mutation of *PROS1*, resulting in the amino acid substitution, Ala525Val, in the SHBG-like domain. It should be noted that this mutation of PS can cause thrombophilia, including severe cerebral infarction in patients with PFO.

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Original Article

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

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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

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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%.

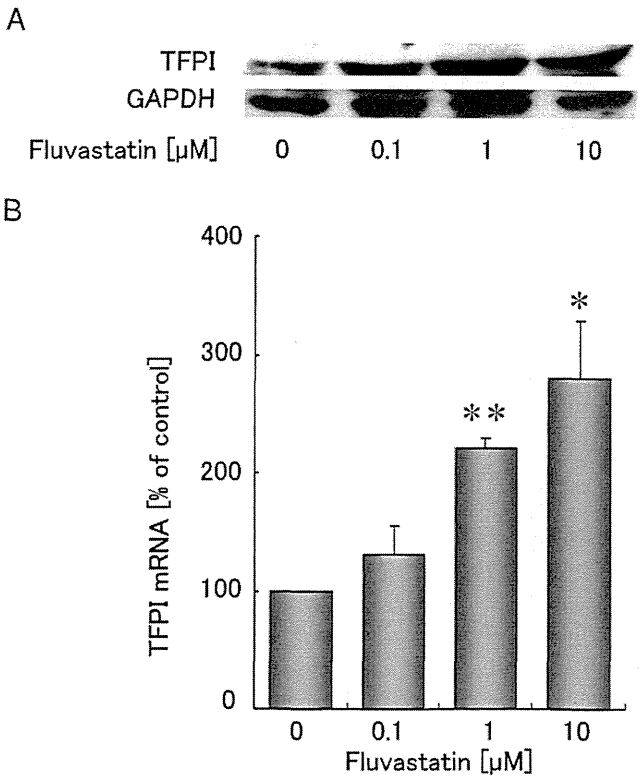

Fig. 1. Fluvastatin upregulates the TFPI antigen and mRNA expression in HUVECs.

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.

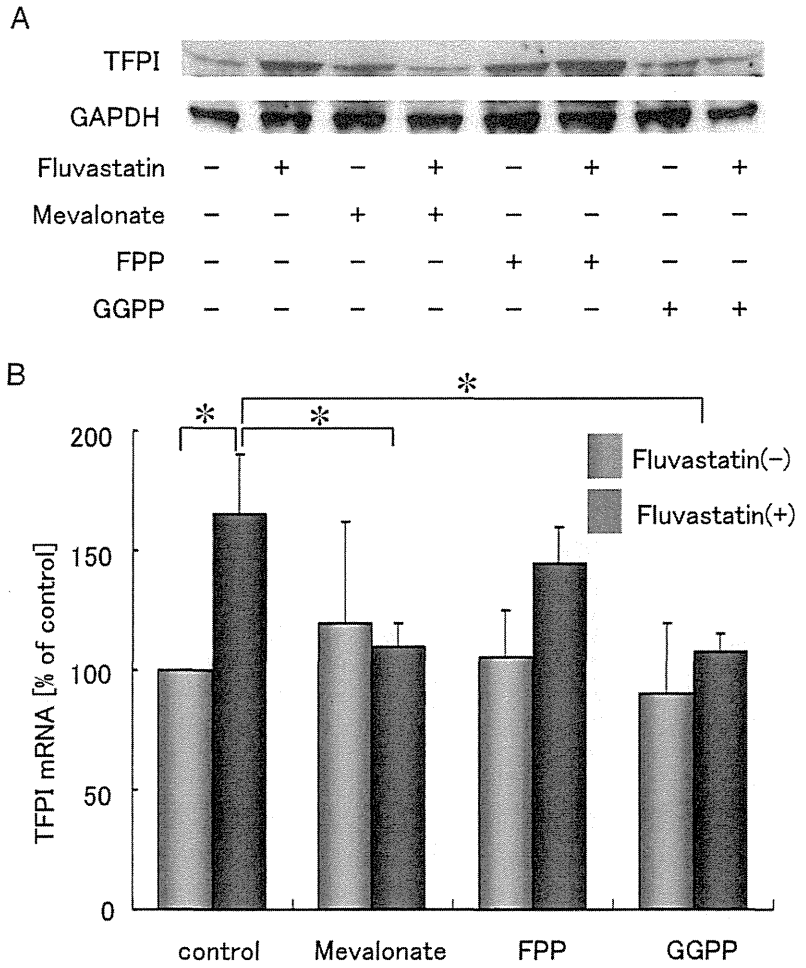

Fig. 2. Fluvastatin-induced upregulation is reversed by mevalonate and GGPP, but not FPP.

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**).

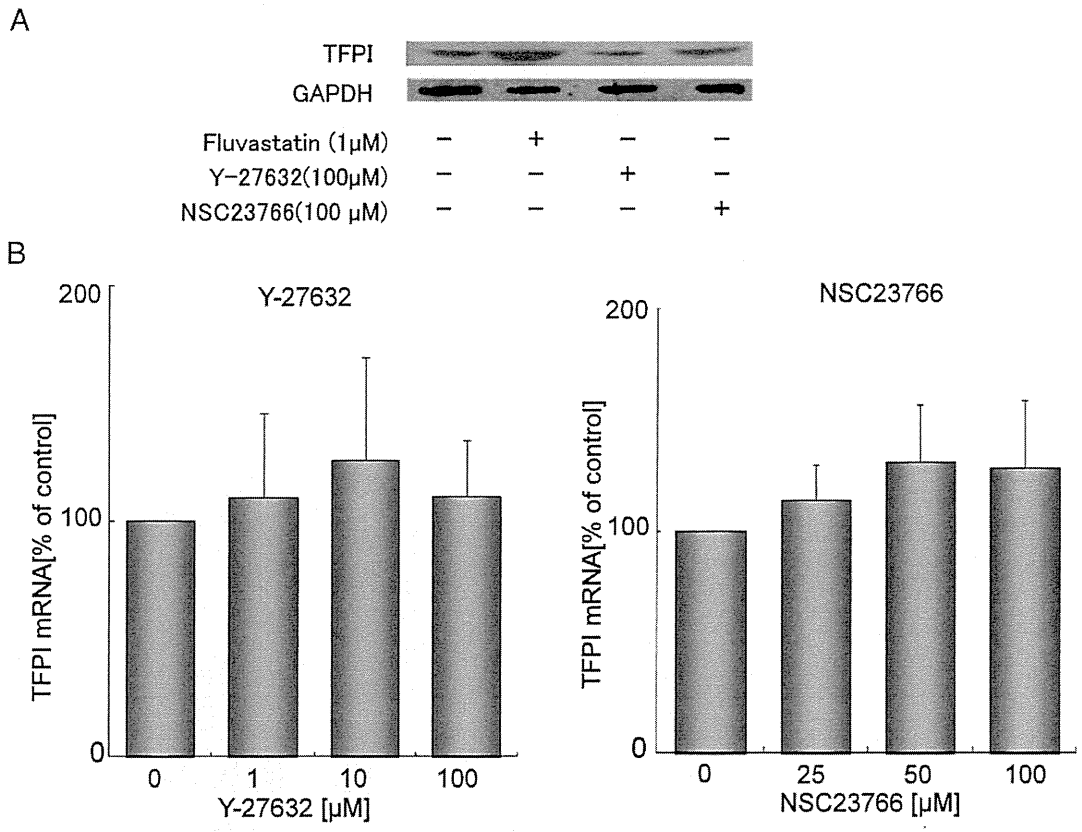

Fig. 3. Y-27632 and NSC23766 have no effect on the fluvastatin-induced upregulation of the TFPI expression in HUVECs.

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

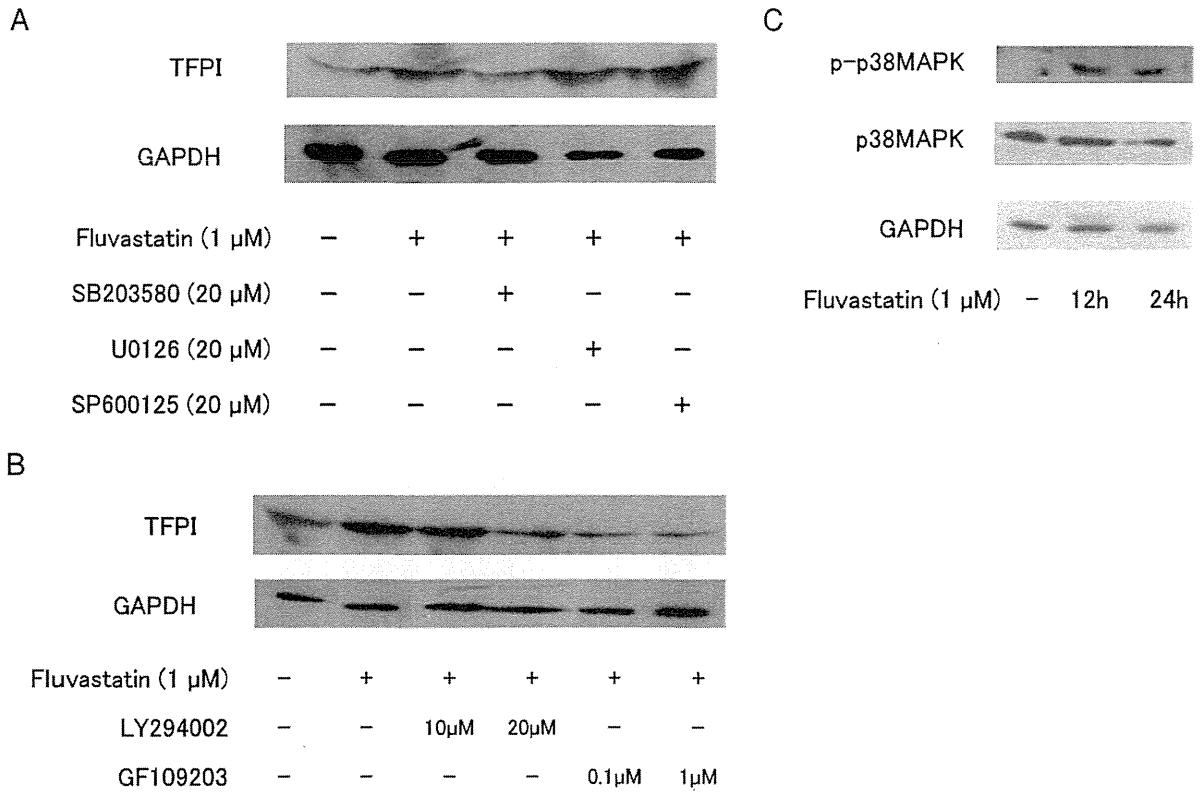

Fig. 4. Fluvastatin-induced TFPI expression is mediated via the activation of p38 MAPK, PKC, and PI3K in HUVECs.

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