Pitavastatin-Induced Thrombomodulin Expression by Endothelial Cells Acts Via Inhibition of Small G Proteins of the Rho Family

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Objective—3-Hydroxyl-3-methyl coenzyme A reductase inhibitors (statins) can function to protect the vasculature in a manner that is independent of their lipid-lowering activity. The main feature of the antithrombotic properties of endothelial cells is an increase in the expression of thrombomodulin (TM) without induction of tissue factor (TF) expression. We investigated the effect of statins on the expression of TM and TF by endothelial cells.

Methods and Results—The incubation of endothelial cells with pitavastatin led to a concentration- and time-dependent increase in cellular TM antigen and mRNA levels. In contrast, the expression of TF mRNA was not induced under the same conditions. A nuclear run-on study revealed that pitavastatin accelerates TM transcription rate. The stimulation of TM expression by pitavastatin was prevented by either mevalonate or geranylgeranylpyrophosphate. Specific inhibition of geranylgeranyltransferase-I and Rac/Cdc42 by GGTI-286 and *Clostridium sordellii* lethal toxin, respectively, enhanced TM expression, whereas inactivation of Rho by *Clostridium botulinum* C3 exoenzyme was ineffective.

Conclusions—Statins regulate TM expression via inhibition of small G proteins of the Rho family; Rac/Cdc42. A statin-mediated increase in TM expression by endothelial cells may contribute to the beneficial effects of statins on endothelial function. (Arterioscler Thromb Vasc Biol. 2003;23:512-517.)

Key Words: endothelium ■ thrombomodulin ■ statins ■ small GTPase

Vascular endothelial cells play an active role in the regulation of blood coagulation and fibrinolysis. The main feature of the antithrombotic properties of endothelial cells is an increase in the expression of thrombomodulin (TM) without induction of tissue factor (TF) expression. TM is a high-affinity receptor for thrombin that is present on endothelial cell surfaces. By forming a complex with thrombin, TM alters the procoagulant activity of thrombin and acts as a cofactor for the thrombin-catalyzed activation of circulating protein C. Activated protein C functions as an anticoagulant via the proteolytic degradation of the coagulation factors Va and VIIIa, thereby limiting the generation of thrombin. Thus, the TM–protein C pathway is a major antithrombotic mechanism present in endothelial cells, and TM is important in regulating the fluidity of circulating blood.

Tissue factor (TF), a membrane glycoprotein receptor that specifically binds factors VII or VIIa, functions as a cellular trigger of the coagulation cascade via activation of factors X and IX. Under normal circumstances, endothelial cells do not display TF activity. Thus, the normal vascular endothelium is relatively inert with respect to initiation of coagulation. A procedure to enhance upregulation of TM without induction of TF expression in cells provides an important clue toward the therapy and prevention of vascular thrombosis.1 Activated protein C also functions as an important modulator of inflammation associated with severe sepsis.2 In vitro data indicate that activated protein C exerts an anti-inflammatory effect by both inhibiting the production of inflammatory cytokines (tumor necrosis factor-α, interleukin-1, and interleukin-6) by monocytes and limiting the rolling of monocytes and neutrophils on injured endothelium via selectin binding.3 Activated protein C indirectly increases the fibrinolytic response by inhibiting plasminogen activator inhibitor. Recently, it has been shown that treatment with activated protein C significantly reduced mortality in patients with severe sepsis.4

Inhibitors of 3-hydroxy-3-methyl coenzyme A (HMG-CoA) reductase (statins) are widely used in the treatment of hypercholesterolemia. Statins act here by interfering with the rate-limiting step of cholesterol biosynthesis, thus effectively lowering the serum levels of cholesterol biosynthesis and...
Figure 1. The mevalonate cascade and their inhibitors. Statins inhibit HMG-CoA reductase and block the synthesis of isoprenoids (FPP/GGPP) and cholesterol. Post-translational geranylgeranylation and farnesylation of small G proteins of the Ras family is an essential prerequisite for their anchoring in the cell membrane and thus for their activity. For the Ras family (Rho, Rac, and Cdc42) geranylgeranylation is the predominant way, whereas Ras proteins are mainly farnesylated. The inhibitors encased with having been square upregulate TM expression, FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; FTI, FTI-276; GGTI, GGTI-286; TcdB, Clostridium difficile Toxin B (TcdB), N-o-nitro-L-arginine methyl ester (L-NAME) and wortmannin were obtained from Sigma. FPP and GGPP were dissolved in methanol/10 mmol/L NH4OH (vol/vol, 7/3). FTI-276, GGTI-286, and Y-27632 were obtained from Calbiochem and Biomedical Technologies Inc. Wortmannin, FTI-276, and GGTI-286 were dissolved in DMSO. The respective solvent in final concentrations used did not affect the expression of either TM or TF. Clostridium botulinum C3 exoenzyme (C3) was obtained from Bi- omol Research Laboratories Inc, and Clostridium sordelli lethal toxin (LT) was kindly provided by K. Aminoto (Kyoto Biken Laboratories, Kyoto 611-0041, Japan). Other all reagents were of analytical grade.

Cell Culture
HUVECs were grown to confluence in HuMedia-EG2 that contained 2% (vol/vol) heat-inactivated fetal bovine serum, 50 µg/mL gentamicin, 50 ng/mL amphotericin B, 10 ng/mL hEGF, 5 ng/mL hFGF-B, 1 µg/mL hydrocortisone, and 10 µg/mL heparin at 37°C in a humidified atmosphere of 5% CO2.

Assay for TM Antigen Levels
HUVECs were plated at a density of 2×10⁵ cells/well in 24-well culture plates. Cells were then incubated with various concentrations of pitavastatin at 37°C for the indicated time. After incubation, the cells were washed 3 times with PBS (pH 7.4). TM was extracted from the cells with 50 mmol/L Tris-HCl (pH 7.4) that contained 0.15 mol/L NaCl, 0.5% Triton X-100, and 1 mmol/L benzamidine hydrochloride. Levels of TM antigen were measured by enzyme immunoassay by using the monoclonal antibodies TM mAb 2, 11, and 20 as previously described.

Quantification of RNA
Total RNA was extracted from cultured cells by using TRIZOL reagent (Life Technologies, Inc). Northern blot analysis was performed as described previously. For the reverse transcriptase-polymerase chain reaction (RT-PCR) studies, single-stranded cDNA was synthesized with 1 µg of total RNA as template by using a SuperScript Preamplification System (Life Technologies, Inc). Quantitative PCR was subsequently performed by continuous fluorescent monitoring of PCR amplification (Light Cycler LC24/Idaho Technology). Real-time PCR amplification was performed in a 20 µL final volume containing 2.5 mmol/L MgCl₂ by using a Quantitect SYBR Green PCR kit (QIAGEN). The amplified PCR fragment for human TM was 146 bp in length, base position 1521 to 1666 (GenBank accession number XM 009595). The amplified PCR fragment for GAPDH, which was used as a “housekeeping” control gene, was 155 bp, base position 436 to 590 (GenBank accession number XM 006959). The standard PCR amplification program included 55 cycles of three steps each, composed of heating at 20°C to 94°C, cooling at 20°C to 55°C, and heating at 20°C to 72°C. Fluorescent product was detected at the last step of each cycle. Levels of TM mRNA were normalized to the concentration of GAPDH mRNA.

Nuclear Run-On Analysis
Nuclear run-on study was performed by using [α-32P]dUTP-labeled RNA of nuclear fraction of cells treated or not treated with 10⁻³ M pitavastatin for 24 hours as described previously. The labeled RNA probe was purified by DNease I and proteinase
K treatments, then absorbed on a nitrocellulose filter, and further purified with trichloroacetic acid washing and additional DNase I treatment. The probe eluted by sodium dodecyl sulfate treatment was recovered by ethanol precipitation. Linearized pCMX and pCMX-TM plasmids, of which the latter was constructed by insertion of the 2.6-kb TM cDNA fragment into HindIII and BamHI sites of pCMX, were absorbed on a Hybond-N filter by using a slot-blotter and fixed by a transilluminator. The filter was incubated with the labeled RNA probe for 36 hours at 42°C in the presence of 40% formamide and 10% dextran sulfate. The intensities of the signals developed by exposure of filters on X-ray films were determined by a Fuji BAS 1500 imaging analyzer (Fuji Photo Film Co., Tokyo, Japan) and expressed relative to the signal of the control band.

Data Analysis
Results of experiments, performed in triplicate, are expressed as mean±SEM. Student t test and one-way ANOVA were used in the statistical analysis when appropriate. The statistical analysis software program, Stat View (Abacus Concept, Inc.) was used. A probability value of <0.05 was accepted as indicating statistical significance.

Results
Statins Upregulate TM Antigen and mRNA Expression by HUVECs at Transcription Level
A significant increase in the level of TM antigen was observed in HUVECs after exposure to pitavastatin, as shown in Figure 2. Incubation with 10^{-5} M pitavastatin for 24 hours enhanced the expression of TM antigen approximately 3-fold compared with that found in unstimulated conditions. Other statins, such as fluvastatin and pravastatin, also increased the level of TM antigen in HUVECs in a dose-dependent manner; however, pravastatin was less effective than either fluvastatin or pitavastatin (Figure 2A). Because the increase in TM antigen levels in response to 10^{-5} M pitavastatin was time-dependent up to 24 hours without evidence of cellular damage (Figure 2B), subsequent experiments were performed by using 10^{-5} mol/L pitavastatin for 24 hours. TM cofactor activity for thrombin-dependent protein C activation was increased in parallel with TM antigen levels (data not shown).

TM and TF mRNA levels were estimated by Northern blot analysis (Figure 3) and by real-time quantitative RT-PCR (Figure I, please see supplemental information at http://atvb.ahajournals.org). Levels of TM mRNA showed a marked concentration- and time-dependent increase after treatment with pitavastatin, being about 3-fold times the control levels at a concentration of 10^{-5} mol/L for 24 hours. There was no evidence of induction of TF mRNA by pitavastatin. To assess the effect of pitavastatin on TM gene transcription rate, a nuclear run-on study was performed on nuclei prepared from cells treated or not
treated with 10^{-5} M pitavastatin for 24 hours. Treatment with pitavastatin increased the TM transcription rate to 350% of the control level (Figure 3C). Furthermore, the addition of actinomycin D mostly abolished the increase in TM mRNA levels mediated by pitavastatin (data not shown). Thus, statins induce TM mRNA levels by increasing TM gene transcription.

**Mevalonate Reverses the Statin-Mediated Induction of TM Expression**

Because statins are inhibitors of HMG-CoA reductase, incubation of cells with these compounds results in a depletion of mevalonate. To test whether statin-mediated induction of TM expression was specific and dependent on mevalonate depletion, HUVECs were incubated with pitavastatin (10^{-5} mol/L) in the presence or absence of mevalonate. As shown in Figure 4, supplementation with mevalonate blocked statin-dependent induction of TM protein (Figure 4) and mRNA expression (Figure III, please see http://atvb.ahajournals.org).

**GGPP Inhibits Statin-Dependent Induction of TM Expression**

FPP and GGPP are important for the post-translational modification of small G proteins of the Ras/Rho family. Prenylation is prerequisite for the activation of these proteins. Ras proteins are predominantly farnesylated, whereas the Rho proteins are mainly geranylgeranylated. To test whether Ras or Rho proteins have a role in the statin-dependent induction of TM expression, HUVECs were incubated with pitavastatin in the presence of the isoprenoid intermediates FPP or GGPP. GGPP reversed the pitavastatin-mediated induction of TM protein (Figure 4) and mRNA (please see http://atvb.ahajournals.org) almost completely. In contrast, FPP was ineffective. The addition of L-NAME, a specific inhibitor of NO synthase, or wortmannin, a phosphatidylinositol 3-kinase (PI3K) inhibitor, did not alter the induction of TM expression by pitavastatin.

**GGTI, TcdB, and LT, But Not C3 or Y-27632, Enhance TM Expression in HUVECs**

The importance of isoprenylation of Rho proteins for the induction of TM was further substantiated by GGTI-286, an inhibitor of the geranylgeranyltransferase-I, and by TcdB, a glucosyltransferase that inactivates the Rho subfamily (Rho, Rac, and Cdc42) without affecting small G proteins of the Ras family. Treatment of HUVECs with either GGTI-286 or TcdB for 24 hours substantially enhanced TM protein (Figure 5) and mRNA levels (Figure III, please see http://atvb.ahajournals.org) in HUVECs, whereas FTI-276, an inhibitor of the farnesyltransferases, was without significant effect. LT is similar to TcdB in that it also possesses monogalactosyltransferase activity. However, these two toxins differ in terms of substrate specificities. Whereas TcdB glucosylates all three Rho family members, Rho is not a substrate for LT, which selectively modifies Rac and, to a lesser extent, Cdc42, as well as some other Ras subfamily members.22 C3 selectively ADP-ribosylates Rho.23 Treatment of HUVECs with LT enhanced TM expression. In contrast, C3, as well as the Rho-associated kinase (ROK) inhibitor, Y-27632, had no effect on TM expression.

**Discussion**

It has previously been shown that inhibition of small GTPases of the Rho/Ras family is important for the cholesterol-independent effects of statins in vascular wall cells. In the present study, we demonstrated the stimulatory effect of pitavastatin, a newly developed HMG-CoA reductase inhibitor, on the expression of TM mRNA and protein in endothelial cells. The expression of TF mRNA was not induced by statin. Fluvastatin similarly increased the level of TM antigen, but pravastatin at the same concentration was less effective than either fluvastatin or pitavastatin. Because pravastatin has a preference toward hepatocytes in terms of cell uptake, the reduced effectiveness of pravastatin against
endothelial cells is probably the result of poor drug accumulation.

Inhibition of protein prenylation by the statins resulted in a striking induction of TM expression. This induction was prevented by mevalonate and GGPP but not by FPP. Furthermore, specific inhibition of geranylgeranyltransferase-I enhanced TM expression, whereas no effect was observed when farnesytransferase was inhibited. Direct inactivation of the Rho subfamily by TcdB also enhanced TM expression. Because Rho proteins are mainly geranylgeranylated, whereas Ras proteins are predominantly farnesylated, these data point to a negative involvement of Rho proteins in TM expression. The mammalian Rho subfamily is composed of RhoA, RhoB, RhoC, Rac1, Rac2, Cdc42, and TC10. Because LT enhanced endothelial TM expression, it is conceivable that Rac or Cdc42 are involved in negatively regulating TM expression (see Figure 1).

Recent studies have demonstrated that some of the pleiotropic effects of statins, other than lowering cholesterol, depend on inhibition of isoprenoid synthesis. It has recently been reported that statins enhance eNOS by stabilizing its mRNA through Rho9 or activating protein kinase Akt through PI3K. However, our results do not suggest the involvement of either NO or PI3K in the induction of TM expression by statins in endothelial cells. Because it has been recently shown that statins activate peroxisome proliferator-activated receptor α (PPARα) through RhoA,24 activated PPARα may contribute to the induction of TM expression. However, this scenario is unlikely because fenofibrate, a PPARα ligand, had no effect on TM expression (data not shown), and RhoA was not involved in regulating TM expression in our system. The physiological significance of the observed increase in TM expression by statins remains unclear. It has been found that cellular and soluble TM can also activate a new potent plasma inhibitor of fibrinolysis, the thrombin-activatable fibrinolysis inhibitor (TAFI; synonymous with plasma procarboxypeptidase B). Through stimulation of TAFI activation, TM downregulates fibrinolysis, which in turn could lead to thrombosis, heart attack, or stroke. However, TM is also a participating cofactor in the activation of protein C. Activated protein C can upregulate fibrinolysis by limiting the activation of TAFI via attenuation of thrombin production. Statin-mediated induction of TM expression might enhance antifibrinolytic activity through TAFI activation. However, this possibility is low because ischemic events were shown to be dramatically suppressed by statin treatment in a variety of clinical studies. Furthermore, it is reported that plasma TAFI concentration is decreased and the fibrinolytic activity index is elevated after statin treatment.27 Recently, it has been reported that recombinant human soluble TM (RHS-TM) prevented the progression of experimental thrombotic glomerulonephritis in rats.28 Furthermore, RHS-TM diminished leukocyte/neutrophil infiltration in these rats. As carboxypeptidase inhibitor significantly diminished the inhibitory effect of RHS-TM on leukocyte/neutrophil infiltration, the observed anti-inflammatory effects of RHS-TM may be caused by the activation of carboxypeptidase. Thus, administration of soluble TM did not result in diminished fibrinolysis, and activation of carboxypeptidase by TM may contribute to its anti-inflammatory action. In contrast, it has been shown that TM deficiency mice exhibit a hypercoagulable state and an increased susceptibility to thrombosis and sepsis.29

It is reported that plasma soluble TM is decreased by statin.27,30,31 Plasma-soluble TM is thought to be generated by endothelial cell damage and is a marker of endothelial cell injury. Thus, our present results showing that statin increases cellular TM expression are not inconsistent with the reduction of plasma soluble TM by statin. Even though 10−7 M pitavastatin significantly increased TM expression, it is uncertain that endothelial TM will be upregulated in vivo by a regular oral dose of statins. The relevance of our findings in vitro must be demonstrated by in vivo experiments. In preliminary experiments, we analyzed the amount of TM in rabbit aortic endothelium by immunostaining with an anti-TM antibody. We used stocked aorta specimens derived from rabbits fed on a cholesterol-rich diet ±0.5 mg/kg pitavastatin. Even the aortic endothelium of control rabbits showed immunostaining with the anti-TM antibody: TM was abundant under basal conditions. We could not distinguish endothelial TM levels between control and pitavastatin-treated rabbits. The use of experimental animals in which endothelial TM expression is reduced in the live state may be necessary.

It has been found that endothelial expression of both endothelial cell protein C receptor and TM was downregulated in coronary atherosclerosis.32 Simvastatin has been found to depress blood clotting by inhibiting the activation of prothrombin, factor V, and factor XIII, as well as by enhancing the inactivation of factor Va.33 Statins also inhibit platelet function, such as reduction in the production of thromboxane A2.34 Animal studies suggest that atorvastatin inhibits platelet deposition on damaged vessels and reduces platelet thrombus formation.35 Moreover, statins have been found to suppress growth of macrophages and macrophage-derived expression of matrix metalloproteinases and TF.14,15 These results combined with our present findings might implicate statins as being potentially beneficial in the prevention of thrombosis and inflammation.

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References


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