Pravastatin Induces Thrombomodulin Expression in TNFα-Treated Human Aortic Endothelial Cells by Inhibiting Rac1 and Cdc42 Translocation and Activity

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Abstract

Expression of functionally active thrombomodulin (TM) on the luminal surface of endothelial cells is critical for vascular thromboresistance. The 3-hydroxyl-3-methyl coenzyme A reductase inhibitor, pravastatin, can protect the vasculature in a manner that is independent of its lipid-lowering activity. We examined the effect of pravastatin on TM expression by human aortic endothelial cells (HAECs) with subsequent tumor necrosis factor α (TNFα) stimulation and investigated the signaling pathways involved. TNFα treatment attenuated TM expression in HAECs in a time-dependent manner. Pravastatin upregulated TM levels in TNFα-treated HAECs. Specific inhibition of geranylgeranyl-transferase-I or the Rho family by GGTI-286 or TcdB, respectively, enhanced TM expression in TNFα-treated HAECs, whereas MAP kinase inhibitors, inactivation of Rho by Clostridium botulinum C3 exoenzyme, or the Rho kinase inhibitor, Y-27632, had no effect. In TNFα-treated HAECs, pravastatin inhibited Rac1 and Cdc42 activation and their translocation to the cell membrane. Blocking the transcriptional activation of NF-κB prevented the TNFα-induced downregulation of TM. The pravastatin-induced increase in TM expression in TNFα-treated HAECs was mediated through inhibition of NF-κB activation. Pravastatin regulates TM expression by inhibiting the activation of the Rho family proteins, Rac1 and Cdc42, and the transcription factor, NF-κB. The increase in endothelial TM activity in response to pravastatin constitutes a novel pleiotropic (nonlipid-related) effect of this commonly used compound and may be of clinical significance in disorders in which deficient endothelial TM plays a pathophysiological role. J. Cell. Biochem. 101: 642–653, 2007.

Key words: endothelial cells; thrombomodulin; pravastatin; Rac1/Cdc42; NF-κB

The 3-hydroxyl-3-methyl coenzyme A (HMG-CoA) reductase inhibitors, known as statins, are effective in lowering the plasma low-density lipoprotein (LDL)–cholesterol concentration and are widely used clinically in the treatment of hypercholesterolemia [Goldstein and Brown, 1990]. Recently, evidence was obtained showing that the beneficial effects of statins go beyond the inhibition of cholesterol biosynthesis [McFarlane et al., 2002]. Moreover, experimental and clinical evidence indicates that the pleiotropic effects of statins include improving endothelial function, enhancing the stability of atherosclerotic plaques, and decreasing oxidative stress, coagulation, and vascular inflammation [Laufs et al., 1998; Undas et al., 2005]. By inhibiting mevalonate synthesis, statins prevent the synthesis of other isoprenoid
intermediates of the cholesterol biosynthetic pathway, such as farnesylpyrophosphate and geranylgeranylpyrophosphate (GGPP). These intermediates are important lipid moieties for the posttranslational modification of proteins, including the γ subunit of heterotrimeric G proteins, heme A, nuclear lamins, and the small GTPases of the Rho and Ras families [Van Aelst and D'Souza-Schorey, 1997]. Because the Rho family is a major target of geranylgeranylation, inhibition of its members may be responsible for many of the cholesterol-independent effects of statins in various cells [Laufs et al., 1998; Masamura et al., 2003].

The vascular endothelium is a major effector compartment for the pleiotropic effects of statins, many of which appear to be related to the ability of statins to prevent the development of endothelial cell dysfunction during various disease states [Callahan, 2003]. It has been shown that statins act directly on endothelial cells. Maintaining an anti-coagulant cell surface is a critical aspect of endothelial function and not only ensures thrombohemorrhagic homeostasis, but also the appropriate regulation of inflammatory and fibroproliferative responses [Zilla et al., 1993]. Statins increase endothelial NO synthase (eNOS) activity in the presence of hypoxia and oxidized LDL, conditions that lead to endothelial dysfunction [Laufs et al., 1997, 1998]. They also inhibit endothelial ET-1 synthesis and plasminogen activator inhibitor expression and induce the secretion of tissue plasminogen activator [Essig et al., 1998; Morikawa et al., 2002]. In addition, statin treatment inhibits expression of the prothrombotic molecule, tissue factor in macrophages of aortic atheroma in Watanabe heritable hyperlipidemic rabbits [Akawa et al., 2001]. The transmembrane glycoprotein, thrombomodulin (TM), plays a particularly important role in maintaining normal endothelial cell function [Esmon, 2003]. TM is expressed on most normal endothelial cells and acts by forming a complex with thrombin, thereby changing its substrate specificity. An inexpensive, safe, and an effective strategy for preventing or reversing endothelial dysfunction by upregulating TM expression would have significant therapeutic potential in such disorders. It is not known whether pravastatin affects TM expression in human aortic endothelial cells (HAECs) exposed to the inflammatory cytokine, tumor necrosis factor α (TNFα). The purpose of this study was to examine the effects of TNFα on TM expression in HAECs, the effect of pravastatin as a potential therapeutic agent, and the mechanisms involved in the effects of pravastatin or TNFα on TM expression. We showed that pravastatin regulates TM expression by inhibiting the activation of the Rho family proteins, Rac and Cdc42, and the transcription factor, NFκB, but not by MAP kinase activation. These findings provided new insights into the molecular mechanisms of action of statins on the vascular wall.

MATERIALS AND METHODS

Materials

Pravastatin was kindly provided by Sankyo Pharma, Inc. (Japan). Clostridium difficile Toxin B (TcdB), GGTI-286, Y-27632, Clostridium botulinum C3 exoenzyme (C3), and parthenolide were obtained from Calbiochem, CA. The final concentrations of the solvents used for the drugs did not affect cell viability.

Culture of HAECs

HAECs were obtained as cryopreserved tertiary cultures from Cascade Biologics (OR) and were grown in endothelial cell growth medium (medium 200, Cascade Biologics) supplemented with 2% fetal bovine serum (FBS), 1 μg/ml of hydrocortisone, 10 ng/ml of human epidermal growth factor, 3 ng/ml of human fibroblast growth factor, 10 μg/ml of heparin, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 1.25 μg/ml of Fungizone (Gibco, NY). The cells were used between passages 3 and 8. The purity of the cultures was verified by staining with monoclonal antibody against human von Willebrand factor [Chen et al., 2002].

Effect of Pravastatin on Cell Viability

HAECs were plated at a density of 10⁴ cells/well in 96-well plates. After overnight growth, the cells were incubated for 24 h with different concentrations of pravastatin, and then cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, MTT (0.5 mg/ml) was applied to the cells for 4 h to allow its conversion into formazan crystals, then after washing with phosphate-buffered saline (PBS), the cells were lysed with dimethyl sulfoxide and the absorbance read at 530 and 690 nm with a DIAS Microplate Reader (Dynex Technologies, Pravastatin Induces TM Expression in TNFα-Treated HAECs 643
The optical density after pravastatin treatment was used as a measure of cell viability and was normalized to that of cells incubated in control medium, which were considered 100% viable.

**Effect of Preincubation With Pravastatin on the Effects of TNFα**

HAECs (10⁶ cells in 5 ml of medium in a 10 cm Petri dish) were incubated with the indicated concentration of pravastatin or medium for the indicated time, then the medium was replaced with fresh medium containing the indicated concentration of TNFα and incubation continued with the indicated time.

**Analysis of Proteins in Cell Lysates**

Western blot analyses were performed as described previously [Chen et al., 2002]. Briefly, a total cell lysate was prepared by lysing cells for 1 h at 4°C in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4 (Cell Signaling, MA), then centrifuging the lysate at 4,000 g for 30 min at 4°C and taking the supernatant. An aliquot of the supernatant (20 μg total protein) was subjected to 10% SDS–PAGE electrophoresis and the proteins transferred onto PVDF membranes (Millipore, MA). To test for the presence of TM, the membranes were incubated with polyclonal rabbit antibodies against human TM (1:1000, Santa Cruz, CA), then with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:5000, Sigma, MO), bound antibody being detected using Chemiluminescence Reagent Plus (NEN, MA). The intensity of each band was quantified using a densitometer. α-tubulin, used as the internal control, was detected using mouse anti-α-tubulin antibody (1:2000, Oncogene, CA) and HRP-conjugated goat anti-mouse IgG antibody (1:5000 dilution, Chemicon, CA). In other studies, the antibodies used were rabbit antibodies against human phospho-JNK, human phospho-p38, human total JNK, or human total ERK1/2, mouse antibodies against human phospho-ERK1/2, and goat antibodies against human total p38 (1:1000, Cell Signaling), followed by HRP-conjugated second antibodies [goat anti-rabbit IgG antibody (1:5000, Sigma) or goat anti-mouse IgG or rabbit anti-goat IgG antibody (1: 5000, Chemicon)], as appropriate.

**Measurement of Rac1 and Cdc42 Translocation**

To prepare membrane and cytosolic proteins, cells were washed with PBS, pelleted, and dissolved in lysis buffer consisting of 1 mM EDTA, 20 mM potassium phosphate buffer, pH 7.0, 0.5 μg/ml of leupeptin, 0.7 μg/ml of pepstatin, 10 μg/ml of aprotinin, and 0.5 mM PMSF on ice, frozen three times at −80°C, and sonicated. The lysates were centrifuged at 29,000g at 4°C for 20 min and the resulting membrane pellet and the supernatant (cytosolic fraction) were stored at −80°C until separated on SDS–PAGE and subjected to Western blotting analysis as described above using monoclonal mouse anti-human Rac1 antibody (1:1000 dilution, Caymen) and HRP-conjugated goat anti-mouse IgG antibody (1:5000 dilution, Chemicon) or polyclonal rabbit anti-human Cdc42 antibodies (1:1000 dilution, Calbiochem) and HRP-conjugated goat anti-rabbit IgG antibody (1:5000 dilution, Santa Cruz).

**Active Rac/Cdc42 Pull-Down Experiments**

HAECs were cultured for 24 h with or without pravastatin, then treated with TNFα, washed with TBS, and lysed by incubation for 3 min at 4°C in Mg²⁺ lysis buffer (Upstate Biotechnology). A sample of clarified lysate (20 μg of protein) was incubated with GST-PBD (p21-binding domain of human PAK-1) and 10 μl of glutathione-Sepharose 4B beads according to the manufacturer’s instructions (Upstate Biotechnology) to precipitate GTP-bound Rac1 or GTP-bound Cdc42 [Benard et al., 1999]. The pelleted beads were washed three times with MLB buffer, suspended in 20 μl of 2× Laemmli sample buffer, and the proteins resolved by 8% SDS–PAGE and immunoblotted using the same antibodies and method as in the previous section. Six percent of the cell lysates were also electrophoresed and immunoblotted to measure the total amount of Rac1 or Cdc42.

**Electrophoretic Mobility Shift Assay (EMSA)**

The preparation of nuclear extracts and the conditions for EMSA reactions have been described previously [Chen et al., 2002]. The 22-mer synthetic double-stranded oligonucleotides used as the NF-κB probe in the gel-shift assay were 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 3′-TCA ACT CCC CTG AAA GGG TCC G-5′.
Statistical Analysis

Values are expressed as the mean ± SEM. Statistical evaluation was performed using one-way ANOVA followed by the Dunnett test, with a P-value < 0.05 being considered significant.

RESULTS

Pravastatin Upregulates TM Expression in TNFα-Treated HAECs

HAECs were incubated for 2, 6, 12, 24, or 48 h with 10 ng/ml of TNFα, and then TM levels in cell lysates were measured on Western blots. TM expression was 89 ± 6%, 88 ± 10%, 62 ± 4%, 51 ± 8%, 19 ± 6%, respectively, in control cells, the reduction with the three longest incubation times being significant (Fig. 1A). When HAECs were pretreated for 24 h with various concentrations of pravastatin before incubation for 24 h with 10 ng/ml of TNFα, pravastatin increased TM levels in a dose-dependent manner (Fig. 1B). Because the increase in TM levels in response to 20 μM pravastatin was time-dependent up to 24 h without evidence of cellular damage (data not shown), subsequent experiments were performed using 20 μM pravastatin for 24 h.

Pravastatin Upregulation of TM Expression in TNFα-Treated HAECs Is Not Dependent on MAPK Phosphorylation

Previous studies have shown that TNFα can activate MAPKs in the signaling pathway leading to cytokine production (Baud and Karin, 2001). In the next set of experiments, we examined whether the effects of pravastatin on TM expression in TNFα-treated cells occurred via the p38, JNK, or ERK1/2 MAPK pathways. As shown in Figure 2A–C, phosphorylation of p38, JNK, and ERK1/2 was significantly increased 20 min after addition of 10 ng/ml of TNFα and this effect was significantly reduced by 24 h pretreatment with 20 μM pravastatin. However, the decrease in TM expression in response to TNFα treatment was not affected by 1 h pretreatment with 30 or 50 μM SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor), or PD98059 (an ERK1/2 inhibitor) (Fig. 2D–F). These results show that, although pravastatin inhibited TNFα-induced MAPK phosphorylation, its effect on TNFα-induced TM expression did not occur via these pathways.

GGTI-286 and TcdB, But Not C3 or Y-27632, Enhance TM expression in HAECs

The importance of isoprenylation of Rho proteins for the induction of TM has been demonstrated using GGTI-286 (a geranylgeranyltransferase-I inhibitor that inhibits the formation of geranylated Rho family proteins), TcdB (a glucosyltransferase that inactivates Rho, Rac, and Cdc42), C3 (a Rho activation
Fig. 2. Pravastatin-induced upregulation of TM expression in TNFα-treated HAECs is not dependent on MAPK phosphorylation. A–C: Western blot analysis of the effect of pravastatin pretreatment on the phosphorylation of p38 (A), ERK1/2 (B), or JNK MAPKs (C) in TNFα-treated HAECs. HAECs were incubated for 24 h with or without 20 μM pravastatin, then the pravastatin was removed and the cells incubated with 10 ng/ml of TNFα for 20 min and aliquots of cell lysate containing equal amounts of protein were subjected to immunoblotting with specific antibodies. D–F: Effects of inhibitors of MAPK phosphorylation on TM expression in control and TNFα-treated HAECs. HAECs were incubated for 1 h with medium or 30 or 50 μM SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor), or PD98059 (an ERK1/2 inhibitor), then the inhibitor was removed and the cells incubated for 24 h with or without 10 ng/ml of TNFα. TM expression was measured by Western blotting. The data are expressed as a percentage of the control value and are the mean ± SEM for three separate experiments. *P < 0.05 compared to untreated cells. †P < 0.05 compared to TNFα-treated cells.
inhibitor), and Y-27632 (a Rho-associated kinase inhibitor) [Masamura et al., 2003]. Incubation of HAECs for 24 h with 10 μM GGTI-286 or 1 ng/ml of TcdB substantially increased TM expression in cells subsequently incubated for 24 h with 10 ng/ml of TNFα (Fig. 3). GGTI-286 or TcdB treatment resulted in the increase of TM expression to 2.2- to 3.0-fold compared with treatment only with TNFα. In contrast, incubation for 24 h with 10 μM Y-27632 or 10 μg/ml of C3 had no effect on TM expression in TNFα-treated cells.

**Pravastatin Inhibits Translocation of Rac1 and Cdc42 in TNFα-Treated HAECs**

The above results showed that the pravastatin-induced upregulation of TM expression was closely associated with Rac1 and Cdc42. The functions of Rac1 and Cdc42 depend on their membrane-associated GTP-binding activity [Van Aelst and D’Souza-Schorey, 1997]. Levels of Rac1 and Cdc42 expression were therefore examined in membrane and cytosolic preparations. As shown in Figure 4A, treatment of HAECs for 30 min with 10 ng/ml of TNFα increased Rac1 membrane levels to 399/6 ± 30%, and decreased Rac1 cytosolic levels to 18 ± 8% of the corresponding levels in control cells. Pretreatment for 24 h with 20 μM pravastatin decreased the TNFα-induced increase in Rac1 membrane levels to 212/6 ± 34% of control levels and increased Rac1 levels in the cytosol to 65 ± 5% of control levels. As shown in Figure 4B, TNFα had a similar effect on Cdc42 distribution (Cdc42 membrane expression increased to 548 ± 18%; Cdc42 cytosolic expression decreased to 17 ± 6%), and pravastatin pretreatment again resulted in a decrease in Cdc42 in the membrane fraction (251 ± 37%) and in an increase in the cytosolic fraction (56 ± 7%). Treatment of HAECs with 10 μM GGTI-286 for 24 h resulted in a substantial decrease in the amount of Rac1 (Fig. 4C) and Cdc42 (Fig. 4D) in the membrane fraction and an increase in the cytosolic fraction in HAECs subsequently treated with TNFα. In contrast, 10 μM Y-27632 had no effect on Rac1 and Cdc42 translocation with TNFα stimulation.

**Pravastatin Inhibits Rac1 and Cdc42 Activation in TNFα-Treated HAECs**

The PAK protein exhibits a selective affinity for the GTP-bound form of Rac1 or Cdc42 [Manser et al., 1994]. HAECs were incubated for 30 min with 10 ng/ml of TNFα, then Rac1 and Cdc42 activation was investigated using the GTP-binding assay. As shown in Figure 5A,B, levels of activated Rac1 and Cdc42 were low in untreated HAECs, but increased to 870/6 ± 25% and 485/6 ± 40% of control levels, respectively, after TNFα treatment and this effect was significantly inhibited by 24 h pretreatment of the HAECs with 20 μM pravastatin (levels of 500/6 ± 23% and 204/6 ± 13% of control levels, respectively).

**Pravastatin Reduces NF-κB Activation in TNFα-Treated HAECs**

A recent study showed that pravastatin reduces the activation of NF-κB, the key factor regulating the induction of many inflammatory cytokines [Dichtl et al., 2003]. Gel-shift assays were performed to determine the effect of pravastatin on NF-κB activation in TNFα-treated HAECs. As shown in Figure 6A, low basal levels of NF-κB binding activity were detected in control cells, and binding was significantly increased by 30 min treatment.
with 10 ng/ml of TNFα. The binding activity was blocked by a 100-fold excess of unlabeled NF-κB probe (data not shown). In pravastatin-pretreated HAECs, the TNFα-induced increase in NF-κB binding was reduced by 30%. As shown on Western blots (Fig. 6B), the inhibitory effect of TNFα on TM levels was overcome by co-incubation of HAECs with TNFα and parthenolide, an NF-κB inhibitor [Sohn et al., 2005]. Treatment of HAECs for 24 h with either 10 μM GGTI-286 or 1 ng/ml of TcdB substantially decreased NF-κB binding activity, whereas 10 μg/ml of C3 or 10 μM Y-27632 had no effect (Fig. 6C).

**DISCUSSION**

In this study, we showed that TNFα significantly decreased TM expression in HAECs in a time-dependent manner. Furthermore, pravastatin increased TM expression in HAECs and pravastatin pretreatment blocked the TNFα-induced downregulation of TM. Treatment with TNFα increased Rac1 and Cdc42 activation and translocation to the cell membrane and these effects were decreased by pravastatin. The inhibition of TM expression in response to TNFα was mediated by NF-κB activation. In contrast, pravastatin-induced upregulation of TM expression was mediated by NF-κB inactivation.

TM on the endothelial cell surface acts as a receptor for thrombin, which activated protein C, providing an anti-coagulant property [Esmon, 2003]. Downregulation of TM impairs normal endothelial function and promotes thrombosis formation [Esmon, 2003]. Our data showed that TNFα significantly reduced TM expression in HAECs in a time-dependent manner. This finding is consistent with previous reports using

![Fig. 4.](image-url)
other types of endothelial cells, that is, EA.Hy926, human umbilical vein, coronary artery, lung, and skin [Shi et al., 2003; Nan et al., 2005]. Based on these results, TM levels influence the degree of thrombosis and provide a measure for assessing the effect of drugs on the thrombotic process. The development of TM enhancers is a major advance in the therapy of thrombotic processes and their use includes the prevention and treatment of disorders. A plethora of pleiotropic statin effects have been reported, including anti-inflammatory, immunomodulatory, and antioxidant effects [Kwak et al., 2000; Carneado et al., 2002; Weitz-Schmidt, 2002]; promotion of osteogenesis [Mundy et al., 1999]; anti-neoplastic effects through inhibition of angiogenesis [Park et al., 2002], tumor cell apoptosis [Wong et al., 2002], tumor progression [Sumi et al., 1994], and metastasis [Kusama et al., 2002]; as well as a large number of effects on the vascular wall and the coagulation and fibrinolytic systems. The vasculoprotective properties have been assumed to result from the ability of statins to down-regulate tissue factor expression in stimulated endothelial cells, to upregulate tissue plasminogen activator and downregulate plasminogen activator inhibitor-I, and to downregulate the expression of adhesion molecules [Essig et al., 1998; Morikawa et al., 2002]. However, these effects are also consistent with an increased ability of the endothelium to activate protein C in situations associated with increased thrombin generation; for example, by increased expression of TM on the endothelial cell surface [Dittman and Majerus, 1990]. Some clinical studies have reported decreased levels of circulating TM fragments in patients on statin therapy [Wada et al., 1993]. A microarray study suggested that statins alter mRNA levels of many genes related to inflammation, vascular constriction, and coagulation, including TM [Morikawa et al., 2002], and another recent report suggested that pitavastatin increases
Here, we showed for the first time that pravastatin increased the levels of TM expression in endothelial cells significantly above that in untreated control cells and also strongly counteracted the effect of TNF-$\alpha$ on TM expression. Our findings suggest the potential use of statin as an adjuvant in patients with sepsis and related disorders.

The MAP kinases, p38, JNK, and ERK1/2, are the central elements of three pathways used by mammalian cells to transduce messages generated by stress agents or growth factors [Dent et al., 2003]. Our study showed that TNF-$\alpha$ caused strong activation of MAPK 3 subtypes in HAECs, as reported in a previous study [Lin et al., 2005]. Pravastatin pretreatment caused a significant reduction in the TNF-$\alpha$-induced phosphorylation of p38, JNK, and ERK. However, a p38 inhibitor (SB203580), a JNK inhibitor (SP600125), or an ERK inhibitor (PD98059) had no significant effect on TM expression in normal or TNF-$\alpha$-treated HAECs. This is the first study to show that, although pravastatin inhibits TNF-$\alpha$-induced MAPK phosphorylation, the pravastatin-induced up-regulation of TM expression in TNF-$\alpha$-treated cells does not involve the MAPK pathways. A previous study showed that the lectin-like domain of TM interferes with neutrophil adhesion to endothelial cells by suppressing adhesion molecule expression by decreasing ERK1/2 activation [Conway et al., 2002]. TM also reduces the thrombin-induced proliferation of human umbilical vein endothelial cells by prolonging the nuclear retention of phosphorylated ERK [Olivot et al., 2001]. These findings may explain why the induction of TM expression was MAPK phosphorylation-independent, whereas the effects of TM on downstream targets were MAPK phosphorylation-dependent.

Recent studies have demonstrated that some of the pleiotropic effects of statins, other than cholesterol lowering, depend on inhibition of protein geranylgeranylation [Masamura et al., 2003]. GGPP, an isoprenoid, provides the lipophilic anchors that are essential for both the membrane attachment and biological activity of the Rho family proteins. Geranylgeranyltransferase-I is responsible for transferring the geranylgeranylated group from GGPP to the Rho family, which results in their activation. The present study demonstrated that specific inhibition of geranylgeranyltransferase-I increased TM expression in TNF-$\alpha$-treated HAECs, showing that the effect of pravastatin on endothelial TM expression predominantly results from deficient protein geranylgeranylation. In addition, it is conceivable that geranylgeranylation of the Rho family is involved in the regulation of TM expression.

The Rho family, which consists of the closely related Rho, Rac1, and Cdc42, has been implicated in the regulation of cellular functions,
including oxidant generation, membrane trafficking, actin cytoskeletal dynamics, and transcription [Etienne-Manneville and Hall, 2002]. It has recently been reported that statins enhance eNOS activity by stabilizing its mRNA through Rho [Laufs et al., 1998] or by activating protein kinase Akt through PI3K [Kureishi et al., 2000]. Simvarstatin prevents endothelial tissue factor induction by inhibition of Rho/Rho-kinase and activation of Akt [Eto et al., 2002]. Inhibition of Rac1 and RhoA by statins reduces myocardial expression of atrial
natriuretic factor and myosin light chain-2, resulting in the development of cardiac hypertrophy [Laufs et al., 2002]. The present study demonstrated that TcdB (an inhibitor of Rho, Rac, and Cdc42), but not C3 (a Rho inhibitor) or Y-27632 (a Rho kinase inhibitor), affects endothelial TM expression. These results suggest that Rac1 and Cdc42, but not Rho, play an important role in TM expression in TNFα-treated HAECs. The difference between the above results may be due to differences in the cell types, cytokines, and inducers used. In the present study, using a specific assay based on the GTPase-binding domain, PAK, we showed that pravastatin inhibited Rac1 and Cdc42 activation in TNFα-treated HAECs. It also inhibited the translocation of Rac1 and Cdc42 to the cell membrane and increased their levels in the cytosol. Our findings suggest that Rac1 and Cdc42 are novel targets for HMG CoA reductase inhibitors, as well as the downstream targets of TM expression in HAECs.

The binding of TNFα to its receptors causes activation of the major transcription factor, NF-κB, which, in turn, induces the expression of genes involved in chronic and acute inflammatory responses [Rothwarf and Karin, 1999]. In the present study, we showed that pravastatin prevented the increase in NF-κB activity caused by TNFα and that blocking NF-κB activation prevented the TNFα-induced downregulation of TM expression. In the present study, NF-κB was found to act as a repressor, in contrast to its normal role as a transcriptional activator. One mechanism by which NF-κB can inhibit gene expression is by competing for the cellular machinery used by other transcriptional factors. NF-κB is a critical mediator of TNFα-induced TM expression and competes for limited pools of the transcriptional co-activator, p300, which is required for TM gene expression [Sohn et al., 2005]. In addition, the present study also demonstrated that NF-κB activation was blocked by TcdB, but not by C3 or Y-27632, suggesting that Rac1 and Cdc42 regulate NF-κB activation. Thus, the stimulatory effect of pravastatin on TM expression is mediated, at least in part, by inhibition of Rac1/Cdc42-dependent NF-κB activation.

In summary, TNFα treatment attenuated TM expression in HAECs. Pravastatin upregulated TM levels in TNFα-treated HAECs. We also showed that the predominant mechanism by which pravastatin upregulates TM involves a decrease in protein geranylgeranylation and the prevention of the activation of Rac1/Cdc42 and NF-κB. These findings demonstrate a novel mechanism by which pravastatin enhances the anti-coagulant and anti-inflammatory properties of the vascular endothelium. These effects of statins may be particularly important in patients with acute coronary syndromes.

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