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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.
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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

Shing-Jong Lin and Yung-Hsiang Chen contributed equally to this work.

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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 [Aikawa 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, NF-κB, 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 pg/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 10^4 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,

VA). 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 (10^6 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 29,000*g* 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 Mg^{2+} 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.

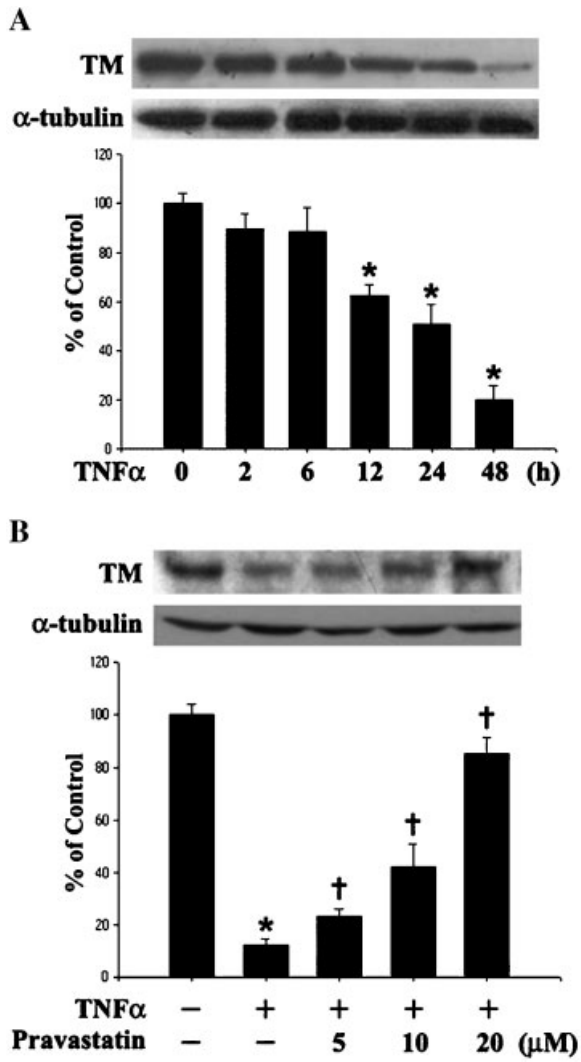

Fig. 1. Pravastatin upregulates TM expression in TNFα-treated HAECs. **A**: HAECs were incubated for 0–48 h with 10 ng/ml of TNFα. **B**: HAECs were incubated for 24 h with various concentrations of pravastatin, then the pravastatin was removed and the cells incubated with 10 ng/ml of TNFα for 24 h. After incubation, TM expression in cell lysates was measured by Western blotting. α-tubulin was used as the loading control. Data are expressed as a percentage of the control value and shown as the mean ± SEM for three separate experiments. $^{*}P<0.05$ compared to untreated (control) cells. $^{\dagger}P<0.05$ compared to TNFα-treated cells.

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

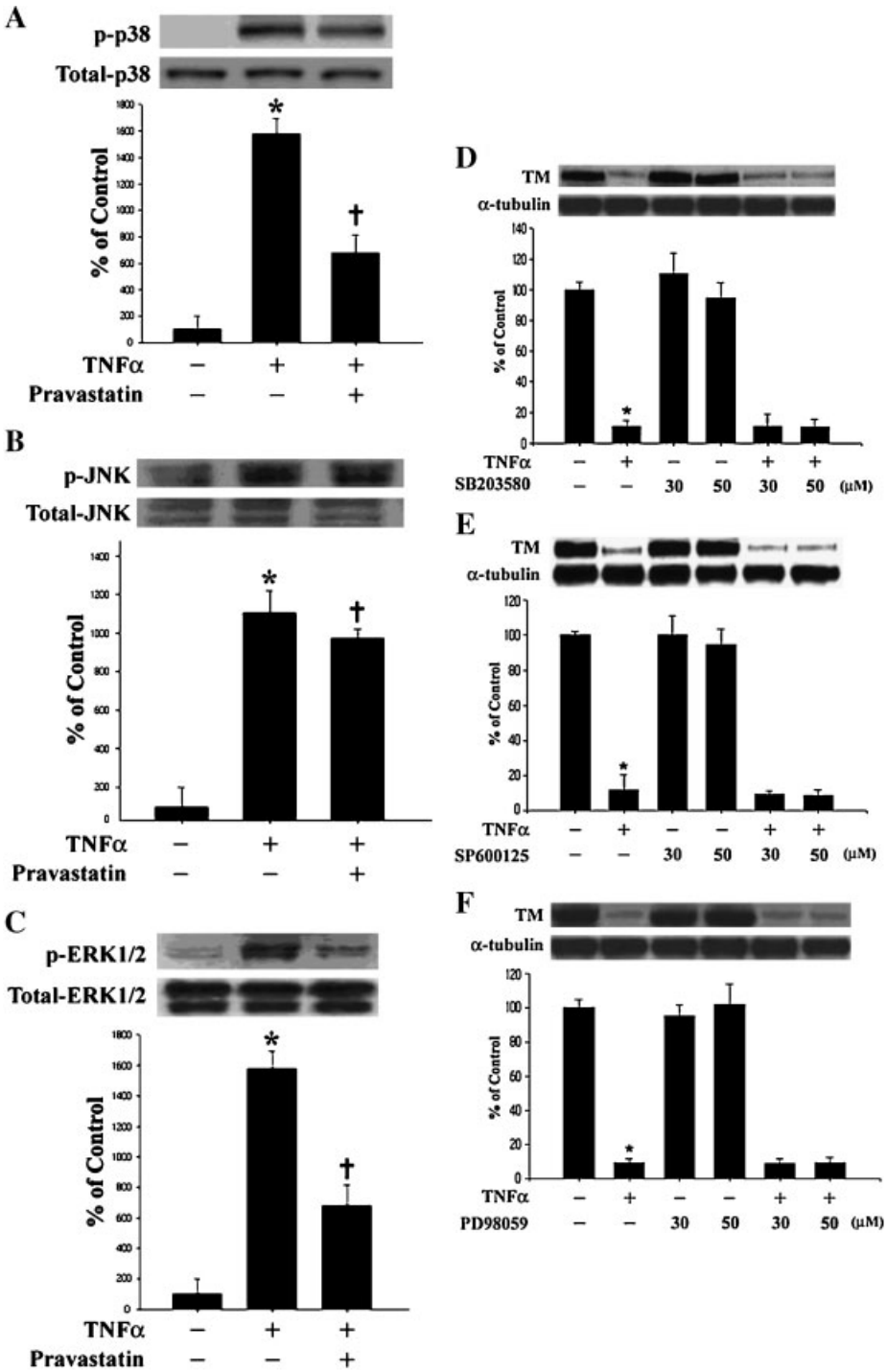

Fig. 2. Pravastatin-induced upregulation of TM expression in TNFα-treated HAECs is not dependent on MAPK phosphorylation. **A**–**C**: Western blot analysis of the effect of pravastatin pretreatment on the phosphorylation of p38 (A), ERK1/2 (B), or JNK MAPKs (C) in TNFα-treated HAECs. HAECs were incubated 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. $^{\dagger}P < 0.05$ 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 ± 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 ± 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.

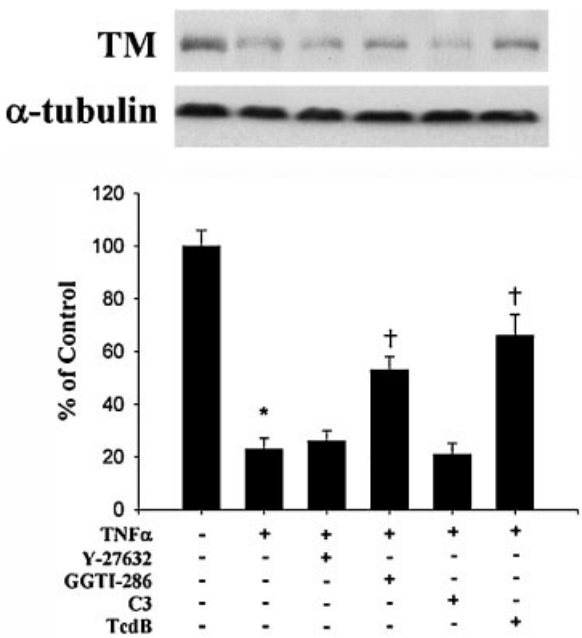

Fig. 3. GGTI-286 and TcdB, but not C3 or Y-27632, increase TM expression in TNFα-treated HAECs. HAECs were incubated for 24 h with or without Y-27632 (10 μM), GGTI-286 (10 μM), C3 (10 μg/ml), or TcdB (1 ng/ml), then the inhibitor was removed and the cells incubated with or without 10 ng/ml of TNFα for 24 h. Aliquots of cell lysate containing equal amounts of protein were then subjected to immunoblotting with anti-TM antibodies. The data are the mean ± SEM for three separate experiments. *$P < 0.05$ compared to untreated cells. †$P < 0.05$ compared to TNFα-treated cells.

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 ± 25% and 485 ± 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 ± 23% and 204 ± 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

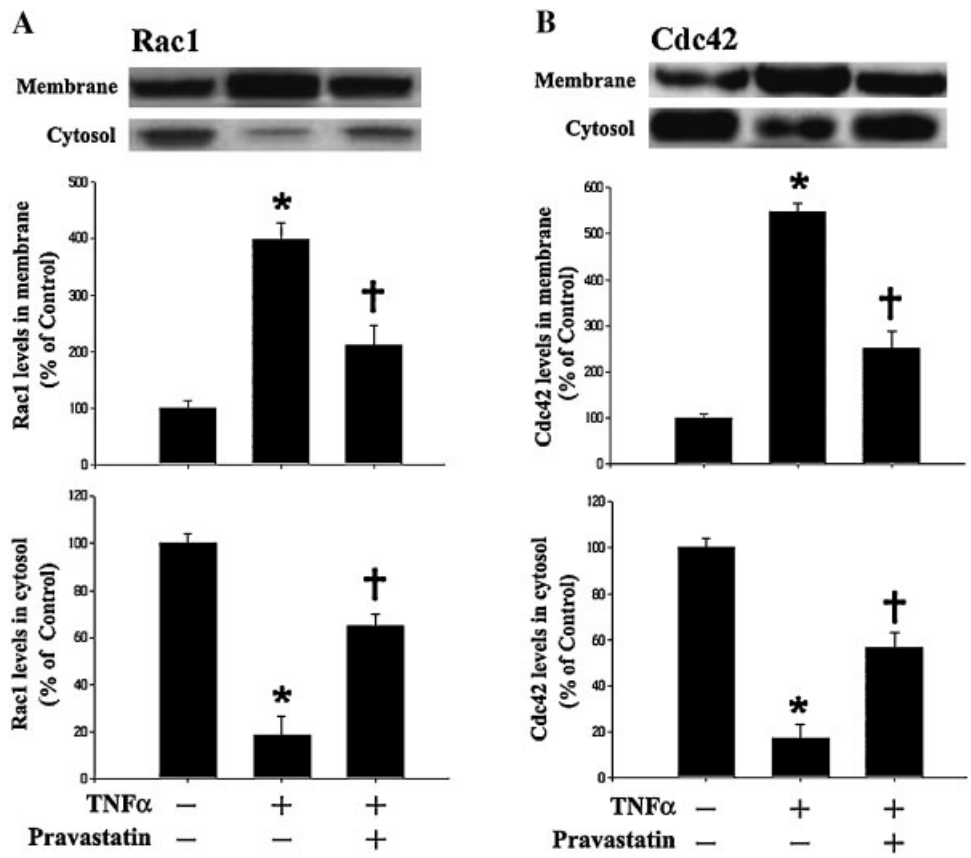

Fig. 4. Translocation of Rac1 and Cdc42 to the cell membrane in TNFα-treated HAECs is inhibited by pravastatin or geranylgeranyltransferase-I. **A** and **B**: Effect of pravastatin. Control cells or cells pretreated for 24 h with 20 μM pravastatin were incubated with or without 10 ng/ml of TNFα for 30 min, then Rac1 (A) and Cdc42 (B) levels were examined in the membrane and cytosolic fractions. **C** and **D**: Effect of geranylgeranyltransferase-I. Control cells or cells pretreated with GGTI-286 (10 μM) or Y-27632 (10 μM) for 24 h were incubated with 10 ng/ml of TNFα for 30 min, then Rac1 (C) and Cdc42 (D) levels were examined in the membrane and cytosolic fractions. $^{*}P < 0.05$ compared to untreated cells. $^{\dagger}P < 0.05$ compared to TNFα-treated cells.

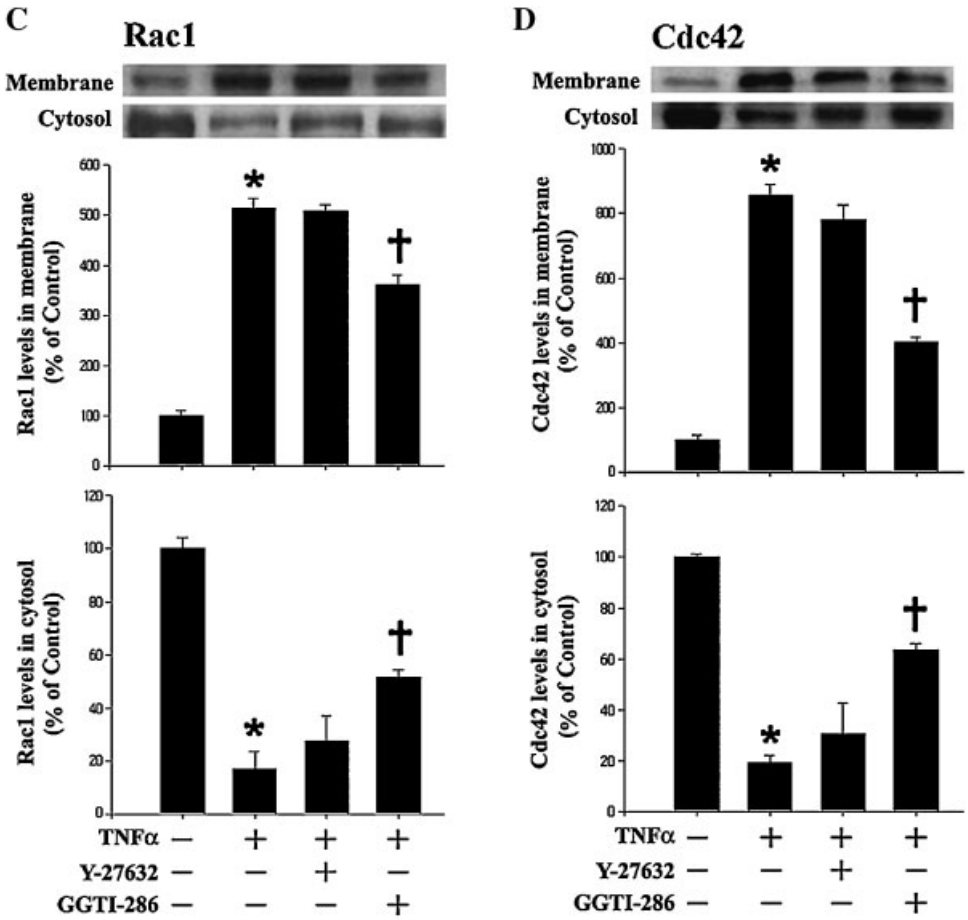

Fig. 4. (*Continued*)

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 downregulate 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

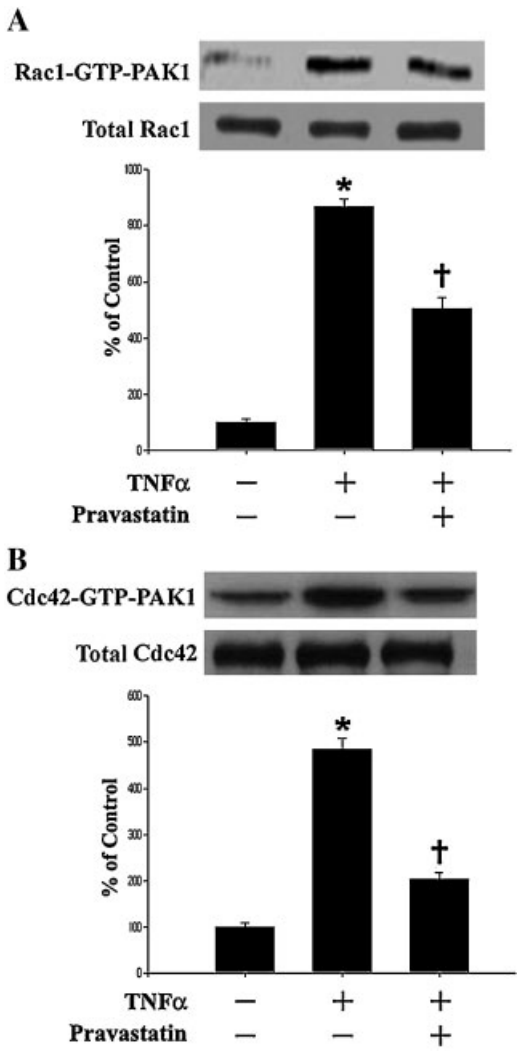

Fig. 5. Pravastatin reduces Rac1 and Cdc42 activity in TNFα-treated HAECs. Active Rac1 (**A**) or Cdc42 (**B**) in HAECs following stimulation with 10 ng/ml of TNFα for 30 min at 37°C. Active Rac1 or Cdc42 was precipitated in a GTP-binding assay and detected following Western blotting with anti-Rac1 or anti-Cdc42 antibodies as described in Materials and Methods. Rac1 and Cdc42 (both active and inactive) present in whole cell lysates are shown in the lower panel as a loading control. $^{*}P < 0.05$ compared to untreated cells. $^{\dagger}P < 0.05$ compared to TNFα-treated cells.

TM expression [Masamura et al., 2003]. 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 TNFα 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 TNFα 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 TNFα-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 TNFα-treated HAECs. This is the first study to show that, although pravastatin inhibits TNFα-induced MAPK phosphorylation, the pravastatin-induced up-regulation of TM expression in TNFα-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 TNFα-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,

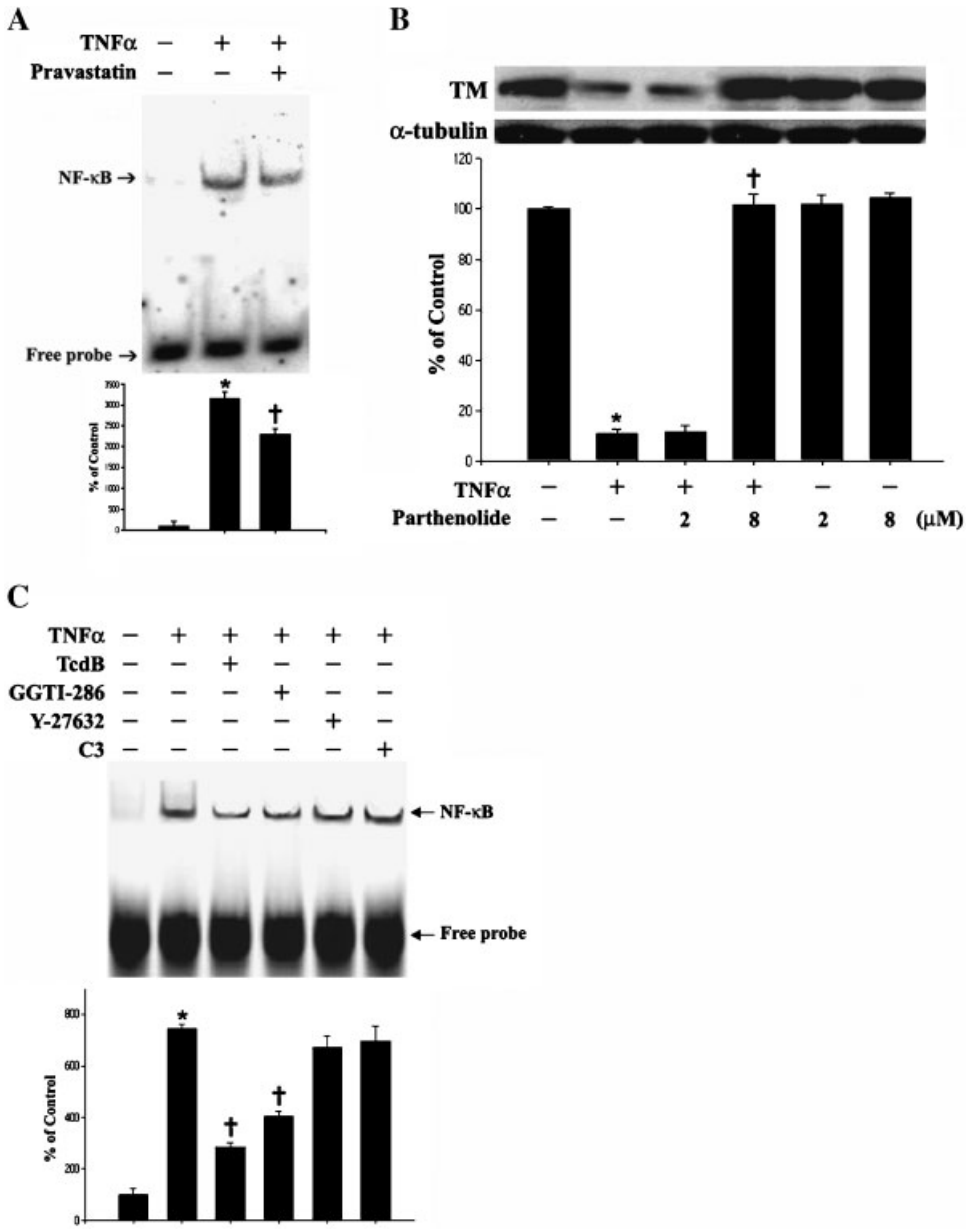

Fig. 6. Pravastatin-induced upregulation of TM expression in TNFα-stimulated HAECs is mediated by inhibition of NF-κB activation. **A**: Nuclear extracts were prepared from untreated cells or from cells with or without pravastatin pretreatment (20 μM, 24 h) subsequently incubated with 10 ng/ml of TNFα for 30 min, and tested for NF-κB DNA binding activity by EMSA. **B**: Cells were co-incubated for 24 h with the indicated concentration of the NF-κB inhibitor, parthenolide, and 10 ng/ml of TNFα, then a cell lysate was prepared and assayed for TM on Western blots. **C**: Cells were incubated for 24 h with 10 μM GGTI-286, 1 ng/ml of TcdB, 10 μM Y-27632, or 10 μg/ml of C3, then for 30 min with 10 ng/ml of TNFα. The nuclear extract was then assayed for NF-κB activation by EMSA. A representative result from three separate experiments is shown and the summarized data for the three experiments are shown in the bar chart. $^{*}P < 0.05$ compared to untreated cells. $^{\dagger}P < 0.05$ compared to TNFα-treated cells.

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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REFERENCES

Aikawa M, Rabkin E, Sugiyama S, Voglic SJ, Fukumoto Y, Furukawa Y, Shiomi M, Schoen FJ, Libby P. 2001. An HMG-CoA reductase inhibitor, cerivastatin, suppresses growth of macrophages expressing matrix metalloproteinases and tissue factor in vivo and in vitro. Circulation 103:276–283.

Baud V, Karin M. 2001. Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol 11:372–377.

Benard V, Bohl BP, Bokoch GM. 1999. Characterization of Rac and Cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. J Biol Chem 274:13198–13204.

Callahan AS. 2003. Vascular pleiotropy of statins: Clinical evidence and biochemical mechanisms. Curr Atheroscler Rep 5:33–37.

Carneado J, Alvarez de Sotomayor M, Perez-Guerrero C, Jimenez L, Herrera MD, Pamies E, Martin-Sanz MD, Stiefel P, Miranda M, Bravo L, Marhuenda E. 2002. Simvastatin improves endothelial function in spontaneously hypertensive rats through a superoxide dismutase mediated antioxidant effect. J Hypertens 20:429–437.

Chen YH, Lin SJ, Chen JW, Ku HH, Chen YL. 2002. Magnolol attenuates VCAM-1 expression in vitro in TNF-alpha-treated human aortic endothelial cells and in vivo in the aorta of cholesterol-fed rabbits. Br J Pharmacol 135:37–47.

Conway EM, Van de Wouwer M, Pollefeyt S, Jurk K, Van Aken H, De Vriese A, Weitz JI, Weiler H, Hellings PW, Schaeffer P, Herbert JM, Collen D, Theilmeier G. 2002. The lectin-like domain of thrombomodulin confers protection from eutrophil-mediated tissue damage by suppressing adhesion molecule expression via nuclear factor kappaB and mitogen-activated protein kinase pathways. J Exp Med 196:565–577.

Dent P, Yacoub A, Fisher PB, Hagan MP, Grant S. 2003. MAPK pathways in radiation responses. Oncogene 22: 5885–5896.

Dichtl W, Dulak J, Frick M, Alber HF, Schwarzacher SP, Ares MP, Nilsson J, Pachinger O, Weidinger F. 2003. HMG-CoA reductase inhibitors regulate inflammatory transcription factors in human endothelial and vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 23: 58–63.

Dittman WA, Majerus PW. 1990. Structure and function of thrombomodulin: A natural anticoagulant. Blood 75: 329–336.

Esmon CT. 2003. Inflammation and thrombosis. J Thromb Haemost 1:1343–1348.

Essig M, Nguyen G, Prie D, Escoubet B, Sraer JD, Friedlander G. 1998. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors increase fibrinolytic activity in rat aortic endothelial cells. Role of geranylgeranylation and Rho proteins. Circ Res 83:683–690.

Etienne-Manneville S, Hall A. 2002. Rho GTPases in cell biology. Nature 420:629–635.

Eto M, Kozai T, Cosentino F, Joch H, Luscher TF. 2002. Statin prevents tissue factor expression in human endothelial cells: Role of Rho/Rho-kinase and Akt pathways. Circulation 105:1756–1759.

Goldstein JL, Brown MS. 1990. Regulation of the mevalonate pathway. Nature 343:425–430.

Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, Walsh K. 2000. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med 6:1004–1010.

Kusama T, Mukai M, Iwasaki T, Tatsuta M, Matsumoto Y, Akedo H, Inoue M, Nakamura H. 2002. 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors reduce human pancreatic cancer cell invasion and metastasis. Gastroenterology 122:308–317.

Kwak B, Mulhaupt F, Myit S, Mach F. 2000. Statins as a newly recognized type of immunomodulator. Nat Med 6:1399–1402.

Laufs U, Fata VL, Liao JK. 1997. Inhibition of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase blocks hypoxia-mediated down-regulation of endothelial nitric oxide synthase. J Biol Chem 272:31725–31729.

Laufs U, La Fata V, Plutzky J, Liao JK. 1998. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation 97:1129–1135.

Laufs U, Kilter H, Konkol C, Wassmann S, Bohm M, Nickenig G. 2002. Impact of HMG CoA reductase inhibition on small GTPases in the heart. Cardiovasc Res 53:911–920.

Lin SJ, Shyue SK, Hung YY, Chen YH, Ku HH, Chen JW, Tam KB, Chen YL. 2005. Superoxide dismutase inhibits the expression of vascular cell adhesion molecule-1 and intracellular cell adhesion molecule-1 induced by tumor necrosis factor-alpha in human endothelial cells through the JNK/p38 pathways. Arterioscler Thromb Vasc Biol 25:334–340.

Manser E, Leung T, Salihuddin H, Zhao ZS, Lim L. 1994. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature 367:40–46.

Masamura K, Oida K, Kanehara H, Suzuki J, Horie S, Ishii H, Miyamori I. 2003. Pitavastatin-induced thrombomodulin expression by endothelial cells acts via inhibition of small G proteins of the Rho family. Arterioscler Thromb Vasc Biol 23:512–517.

McFarlane SI, Muniyappa R, Francisco R, Sowers JR. 2002. Pleiotropic effects of statins: Lipid reduction and beyond. J Clin Endocrinol Metab 87:1451–1458.

Morikawa S, Takabe W, Mataki C, Kanke T, Itoh T, Wada Y, Izumi A, Saito Y, Hamakubo T, Kodama T. 2002. The effect of statins on mRNA levels of genes related to inflammation, coagulation, and vascular constriction in HUVEC. Human umbilical veinendothelial cells. J Atheroscler Thromb 9:178–183.

Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G. 1999. Stimulation of bone formation in vitro and in rodents by statins. Science 286:1946–1949.

Nan B, Lin P, Lumsden AB, Yao Q, Chen C. 2005. Effects of TNF-alpha and curcumin on the expression of thrombomodulin and endothelial protein C receptor in human endothelial cells. Thromb Res 115:417–426.

Olivot JM, Estebanell E, Lafay M, Brohard B, Aiach M, Rendu F. 2001. Thrombomodulin prolongs thrombin-induced extracellular signal-regulated kinase phosphorylation and nuclear retention in endothelial cells. Circ Res 88:681–687.

Park HJ, Kong D, Iruela-Arispe L, Begley U, Tang D, Galper JB. 2002. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors interfere with angiogenesis by inhibiting the geranylgeranylation of RhoA. Circ Res 91:143–150.

Rothwarf DM, Karin M. 1999. The NF-kappa B activation pathway: A paradigm in information transfer from membrane to nucleus. Sci STKE 1999:RE1.

Shi J, Wang J, Zheng H, Ling W, Joseph J, Li D, Mehta JL, Ponnappan U, Lin P, Fink LM, Hauer-Jensen M. 2003. Statins increase thrombomodulin expression and function in human endothelial cells by a nitric oxide-dependent mechanism and counteract tumor necrosis factor alpha-induced thrombomodulin downregulation. Blood Coagul Fibrinolysis 14:575–585.

Sohn RH, Deming CB, Johns DC, Champion HC, Bian C, Gardner K, Rade JJ. 2005. Regulation of endothelial thrombomodulin expression by inflammatory cytokines is mediated by activation of nuclear factor-kappa B. Blood 105:3910–3917.

Sumi S, Beauchamp RD, Townsend CM, Pour PM, Ishizuka J, Thompson JC. 1994. Lovastatin inhibits pancreatic cancer growth regardless of RAS mutation. Pancreas 9: 657–661.

Undas A, Brummel-Ziedins KE, Mann KG. 2005. Statins and blood coagulation. Arterioscler Thromb Vasc Biol 25:287–294.

Van Aelst L, D'Souza-Schorey C. 1997. Rho GTPases and signaling networks. Genes Dev 11:2295–2322.

Wada H, Mori Y, Kaneko T, Wakita Y, Nakase T, Minamikawa K, Ohiwa M, Tamaki S, Tanigawa M, Kageyama S, Deguchi K, Nakano T, Shirakawa S, Suzuki K. 1993. Elevated plasma levels of vascular endothelial cell markers in patients with hypercholesterolemia. Am J Hematol 44:112–116.

Weitz-Schmidt G. 2002. Statins as anti-inflammatory agents. Trends Pharmacol Sci 23:482–486.

Wong WW, Dimitroulakos J, Minden MD, Penn LZ. 2002. HMG-CoA reductase inhibitors and the malignant cell: The statin family of drugs as triggers of tumor-specific apoptosis. Leukemia 16:508–519.

Zilla P, von Oppell U, Deutsch M. 1993. The endothelium: A key to the future. J Card Surg 8:32–60.