Atorvastatin induces thrombomodulin expression in the aorta of cholesterol-fed rabbits and in TNFα-treated human aortic endothelial cells

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Summary. Expression of functionally active thrombomodulin (TM) on endothelial cells is critical for vascular thromboresistance. 3-Hydroxyl-3-methyl coenzyme A reductase inhibitors (statins) can protect the vasculature from inflammation and atherosclerosis caused by cholesterol-dependent and cholesterol-independent mechanisms. In the present study, the effects of atorvastatin on TM expression in the aorta of cholesterol-fed rabbits and in TNFα-treated human aortic endothelial cells (HAECs) were investigated. When rabbits were fed a 0.5% cholesterol diet with and without supplementation with atorvastatin for 9 weeks, the neointimal area in the thoracic aorta of the atorvastatin-treated group was significantly reduced and there was significant induction of TM protein expression. In HAECs, TNFα treatment decreased the expression of TM in a time- and dose-dependent manner and atorvastatin pretreatment upregulated the expression of TM mRNA and protein in HAECs with or without TNFα treatment. Atorvastatin also inhibited monocyte adhesion to control and TNFα-treated HAECs via TM expression. ERK1/2 phosphorylation was significantly reduced by 24 h pretreatment with atorvastatin, whereas TNFα increased the phosphorylation of the MAPKs, p38, JNK, and ERK1/2. Blocking the transcriptional activation of NF-κB and nuclear translocation of NF-κB p65 prevented the TNFα-induced downregulation of TM. Atorvastatin regulated TM expression in control and TNFα-treated HAECs by inhibiting the activation of ERK and NF-κB. The increase in endothelial TM activity in response to atorvastatin constitutes an important pleiotropic effect of this commonly used compound and may be of clinical significance in cardiovascular disorders in which deficient endothelial TM plays a pathophysiological role.

Key words: Atorvastatin, Thrombomodulin, Endothelial cells, Cholesterol-fed rabbit, MAPKs

Introduction

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 (Shepherd et al., 1995). They currently constitute the most widely prescribed class of drugs for the reduction of morbidity and mortality associated with cardiovascular diseases. Interestingly, many of these drugs have clinically beneficial pleiotropic effects unrelated to their lipid-lowering effects (Yamakuchi et al., 2005; Devaraj et al., 2006). Statins have been shown to improve endothelial dysfunction, enhance the stability of atherosclerotic plaques, and decrease oxidative stress, coagulation, and vascular inflammation (Calabro and Yeh, 2005; Undas et al., 2005). However, the anti-atherosclerotic effect and the related mechanism of atorvastatin on cholesterol diet-induced atherosclerosis in a rabbit model and tumor necrosis factor α (TNFα)-treated endothelial cells have not been systemically examined.

Several studies have demonstrated anti-inflammatory effects of statins on various cell types implicated in atherosclerosis, including endothelial cells (Seljeflot et al., 2002). Endothelial cells play a central role in the atherosclerotic process and are closely related

to cardiovascular disorders (Ross, 1993). Hyper-cholesterolemia and oxidized LDL-cholesterol impair endothelial function by changing endothelium-dependent vasoreactivity and by increasing inflammatory and thrombogenic responses. The vascular endothelium may be a major target for the pleiotropic effects of statins, many of which appear to be related to their ability to improve the vasodilatory properties of a dysfunctional endothelium and also to change it from a prothrombotic to a thromboresistant state (Rosenson and Tangney, 1998). Thrombomodulin (TM), a transmembrane glycoprotein, plays a particularly important role in maintaining normal endothelial cell function (Esmon, 2003). TM is expressed on normal endothelial cells and acts by forming a complex with thrombin, thereby inhibiting coagulation (Medina et al., 2007). An inexpensive, safe, and effective strategy for preventing or reversing endothelial dysfunction by upregulating TM expression would have significant therapeutic potential in cardiovascular disorders. It is not known whether atorvastatin affects TM expression in untreated human aortic endothelial cells (HAECs) or HAECs exposed to the inflammatory cytokine, TNFα, or in cholesterol-fed rabbits. To elucidate the molecular mechanisms and identify the potential loci for therapeutic intervention, we employed in the present study a two-pronged approach with an *in vivo* system of stimulating hypercholesterolemia by feeding New Zealand white rabbits a cholesterol diet, and a more simplistic in vitro approach of eliciting inflammation, a key step in atherosclerosis, in cultured human aortic endothelial cells by the TNFα and the effects of atorvastatin treatment on these systems, focusing on TM expression and the relevant signal transduction pathways. We showed that atorvastatin upregulates TM expression by inhibiting activation of the MAPK, ERK, and the transcription factor, NF-κB. These findings provide new insights into the molecular mechanism of action of statins on the vascular wall.

Materials and methods

Animal care and experimental procedures

Sixty male New Zealand white rabbits (2.5-3.0 Kg) were used. The experimental procedures and animal care and handling conformed to the guidelines for animal care of the National Taiwan University. The animals were placed on a 0.5% cholesterol diet (Purina Mills Inc., MO, USA) and randomly allocated to one of two groups: (1) a group with no drug treatment (n=24); (2) an experimental group treated by oral ingestion of atorvastatin (2.5 mg/Kg/day) (n=24). The dose of atorvastatin used was based on published work (Aragoncillo et al., 2000). Twelve age-matched male rabbits on the regular diet were used as controls. After 3 or 9 weeks on the diet, the rabbits were euthanized by intravenous injection of 35-40 mg/kg of sodium pentobarbital and the thoracic aorta was dissected and cut into five segments. A small part of each arterial

segment was taken, immersion-fixed with 4% buffered paraformaldehyde, paraffin-embedded, then cross-sectioned for morphometry and immunohistochemistry, while the remaining larger portion was frozen in liquid nitrogen for protein isolation.

Biochemical measurements

Blood samples for biochemical measurements were collected from each animal before, and at 3 and 9 weeks after, the start of the cholesterol diet. Serum total cholesterol and triglyceride were measured using Merck assay kits (Parmstadt, Germany). Serum glucose, blood urea nitrogen, creatinine, glutamic-oxalacetic transaminase, glutamic-pyruvic transferase, and γ-glutamyl transferase were also measured.

Hematoxylin-eosin staining, morphometry, and immunohistochemistry

One 5 µm thick cross-section was taken from each segment of the thoracic aorta and stained with hematoxylin and eosin. Morphometric analysis of the intimal area of 5 arterial cross-sectional areas per animal was performed using Image-Pro Plus 4.5. To identify the cell type showing TM expression, four serial sections were examined by immunohistochemistry for, respectively, TM, endothelial cells, smooth muscle cells, and macrophages. In negative controls, the primary antibodies were omitted.

Culture of HAECs and U937 cells

HAECs were obtained as cryopreserved tertiary cultures from Cascade Biologics (OR, USA) and were grown in endothelial cell growth medium (medium 200, Cascade Biologics) supplemented with LSGS (Cascade Biologics). The cells were used between passages 3 and 8 and did not show any abnormal morphology.

U937 cells, originally derived from a human histiocytic lymphoma and obtained from the American Type Culture Collection (MD, USA), were grown in RPMI 1640 medium (M.A. Bioproducts, MD, USA) containing 10% FBS.

Effect of atorvastatin and TNFα 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 atorvastatin or TNFα, then cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Western blotting

The thoracic aortas were homogenized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 0.05% SDS, 0.5% Triton X-100, and 1 mM PMSF, pH 7.4). The proteins were applied to 10% SDS–PAGE and

electroblotted onto polyvinylidene difluoride (PVDF) membranes (NEN), which were then incubated for 1 h at room temperature with goat anti-rabbit TM antibody (1:1,000 dilution, American Diagonostica, CT, USA), washed, and incubated for 1 h at room temperature with HRP-conjugated mouse anti-rabbit IgG monoclonal antibody (1:3,000, Sigma).

HAECs (10^6 cells) were lysed with lysis buffer as above. To test for the presence of TM, the membranes were incubated with polyclonal rabbit antibodies against human TM (1:1000, Santa Cruz, CA, USA), then with HRP-conjugated goat anti-rabbit IgG antibody (1:3000, Santa Cruz), bound antibody being detected using Chemiluminescence Reagent Plus (NEN, MA, USA). α-tubulin, used as the internal control, was detected using mouse anti-α-tubulin antibody (1:2000, Oncogene, CA, USA) and HRP-conjugated goat anti-mouse IgG antibody (1:5000, Chemicon, CA, USA). In other studies, the antibodies used were rabbit antibodies against human phospho-JNK, human phospho-p38, human total JNK, human total ERK1/2, or human total p38, mouse antibodies against human phospho-ERK1/2 (1:1000, Cell Signaling), followed by HRP-conjugated second antibodies [goat anti-rabbit IgG antibody (1:5000, Sigma) or goat anti-mouse IgG (1:5000, Chemicon)], as appropriate.

Quantitative Real Time-PCR

Real-time PCR was performed using a Stratagene Mx3000P® real time PCR system. Primers for TM were 5'-GACGTGGATGACTGCATACTG-3' and 5'-TACTCGCAGTTGGCTCTGAAG-3'. For ß-actin the primers were 5'-CTGGACTTCGAGCAAGAGATG-3' and 5'-TGATGGAGTTGAAGGTAGTTTCG-3'.

Thrombomodulin activity assay

Cells were incubated with 40 μL of reaction mixture (37.5 nM thrombin and 5 μg/mL protein C in the washing buffer) at 37°C for 30 min. Protein C activation was terminated by adding 40 μL of antithrombin III (6 IU/mL, Calbiochem Novabiochem, CA, USA) and heparin (12 IU/mL). The enzymatic activity of activated protein C was measured with the peptide substrate H-D-Lys-Z-Pro-Arg-4-nitroanilidediacetate (Chromozym PCa; 0.5 mM in 20 mM Tris, pH 7.4, 0.15 M NaCl, and 5 mg/mL bovine serum albumin) at 37°C. The absorbance change at 405 nm was measured with a Kinetic assay reader (Molecular Devices, SPECTRA MAX 340). Some wells containing thrombin and protein C in the absence of cells were treated similarly and used as the control blank.

Immunocytochemical Localization of NF-κB p65

To examine NF-κB expression *in situ*, confluent HAECs (controls or cells treated for 24 h with 10 μM atorvastatin) were exposed to TNF-α (10 ng/mL) for 30 min, and then reacted for 1 h at room temperature with mouse anti-human NF-κB p65 antibody (1:500, Transduction, KY, USA). After washes, the slides were incubated for 1 h at 37°C with FITC-conjugated goat anti-mouse IgG, and then viewed on a fluorescent microscope.

Endothelial cell-leukocyte adhesion assay

U937 cells were labeled for 1 h at 37°C with 10 μM BCECF/AM (Boehringer-Mannheim, Mannheim, Germany) in serum-free RPMI 1640 media. Labeled U937 cells (10^6 cells) were added to each HAEC-containing well and incubation continued for 1 h, and the degree of U937 cell adhesion to HAECs was evaluated on an ELISA reader after lysing the cells with lysis buffer.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)

The preparation of nuclear protein extracts and the conditions for the EMSA 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
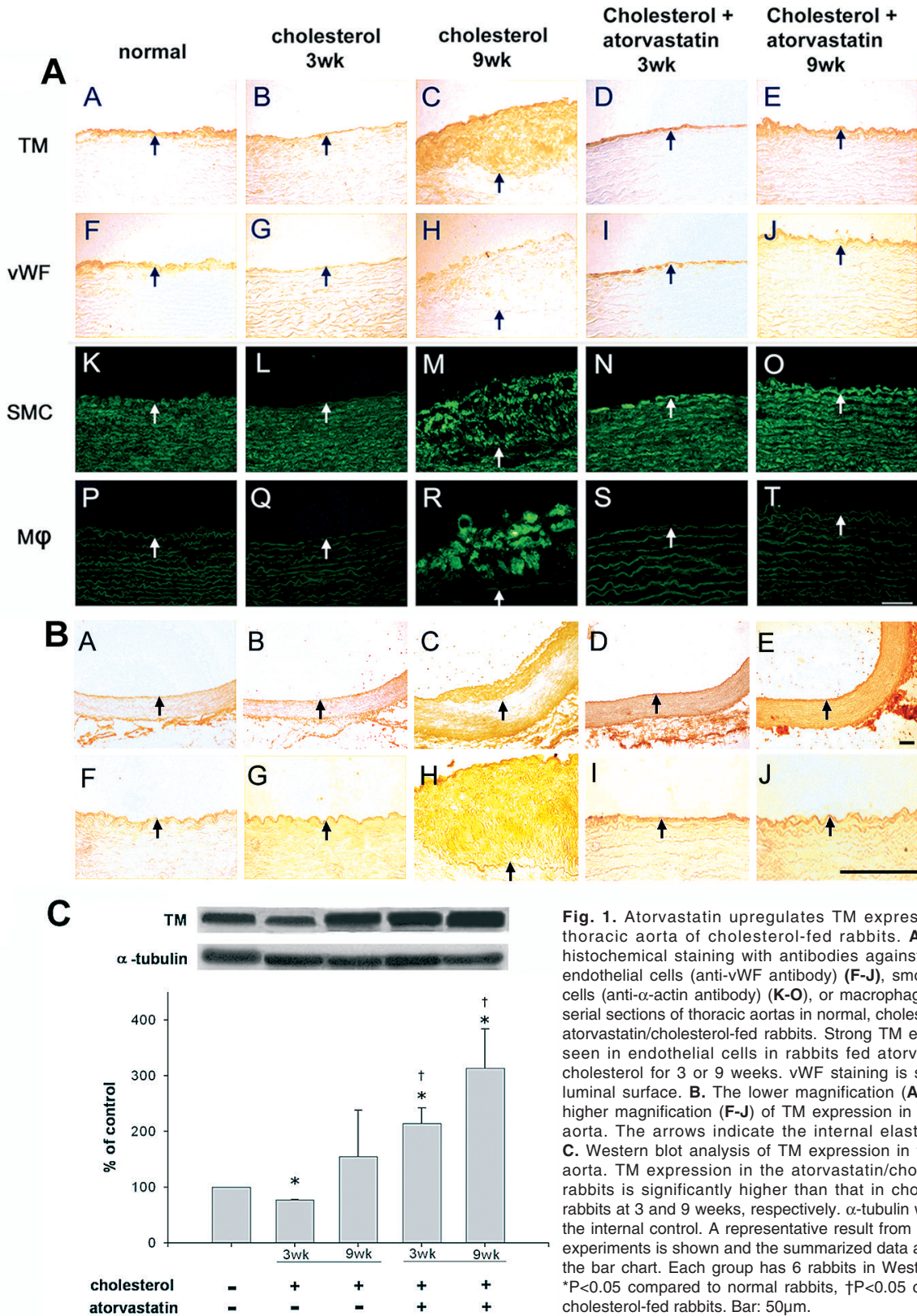
Values are expressed as the mean±SEM. Statistical evaluation was performed using one-way ANOVA followed by the Dunnett test, with a p value <0.05 being considered significant.

Results

Atorvastatin decreases atherosclerotic lesions and increases TM expression in the thoracic aorta of cholesterol-fed rabbits

Over the experimental period, there were no differences in weight gain and final weight between the normal, cholesterol-fed, and atorvastatin-treated, cholesterol-fed rabbits. There were also no significant differences in levels of glucose, aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, or creatinine or any other biochemical parameters. In the control group, the plasma cholesterol and triglyceride concentrations before the experiment were 54±3 and 60±5 mg/dL, respectively, and did not change significantly during the 9-week feeding period (58±4 and 63±6 mg/dL at 9-weeks for cholesterol and triglyceride, respectively, n=12). In the cholesterol-fed group, total plasma cholesterol and triglyceride levels increased to 1648±93 and 198±30 mg/dL, respectively, while the corresponding levels in the atorvastatin-treated animals were significantly lower at 1016±86 and 104±22 mg/dL, respectively. Morphometric analysis showed that the intimal area in the atorvastatin-treated group was

Fig. 1. Atorvastatin upregulates TM expression in the thoracic aorta of cholesterol-fed rabbits. **A.** Immuno-histochemical staining with antibodies against TM **(A-E)**, endothelial cells (anti-vWF antibody) **(F-J)**, smooth muscle cells (anti-α-actin antibody) **(K-O)**, or macrophages (**P-T**) on serial sections of thoracic aortas in normal, cholesterol-fed, or atorvastatin/cholesterol-fed rabbits. Strong TM expression is seen in endothelial cells in rabbits fed atorvastatin and cholesterol for 3 or 9 weeks. vWF staining is seen on the luminal surface. **B.** The lower magnification (**A-E**) and the higher magnification (**F-J**) of TM expression in the thoracic aorta. The arrows indicate the internal elastic laminae. **C.** Western blot analysis of TM expression in the thoracic aorta. TM expression in the atorvastatin/cholesterol-fed rabbits is significantly higher than that in cholesterol-fed rabbits at 3 and 9 weeks, respectively. α-tubulin was used as the internal control. A representative result from six separate experiments is shown and the summarized data are shown in the bar chart. Each group has 6 rabbits in Western blotting. *P<0.05 compared to normal rabbits, †P<0.05 compared to cholesterol-fed rabbits. Bar: 50μm.

significantly less than that in the cholesterol-fed group [(165±30) x10^3 µm^2 versus (680±50) x10^3 µm^2)].

To study the effect of atorvastatin on TM expression in cholesterol-fed rabbits, immunohistochemical staining with antibodies against TM, endothelial cells, smooth muscle cells, or macrophages was carried out on serial sections (Fig. 1A). In the cholesterol-fed group, vWF staining was seen on the luminal surface of the thoracic aorta and TM staining was seen on the luminal surface and the markedly thickened intima at 9 weeks. The thickened intima was mainly composed of macrophages and smooth muscle cells. In the atorvastatin-treated animals, the intimal area was reduced, but vWF staining was present on the luminal surface and strong TM expression was present in endothelial cells at 3 and 9 weeks. In the normal group, the intima was very thin and smooth muscle cells were only detected in the tunica media, while the luminal surface showed only faint staining for TM (Fig. 1B). In addition, Western blot analysis showed that rabbits with cholesterol diet for 3 weeks significantly reduced TM expression compared with the control group, whereas rabbits with cholesterol-fed diet for 9 weeks showed a trend to increase TM expression (Fig. 1C). Furthermore, TM expression was significantly higher in atorvastatin-treated cholesterol-fed rabbits than cholesterol-fed rabbits at 3 and 9 weeks.

Atorvastatin upregulates TM mRNA and protein levels in control and TNFα-treated HAECs

HAECs were incubated with 10 µM atorvastatin for 2, 6, 12, 24, or 48 h, then TM levels in cell lysates were measured using Western blots. A significant increase in TM levels was seen at 6, 12, 24, and 48 h (Fig. 2A). When HAECs were incubated for 24 h with various concentrations of atorvastatin and TM levels in cell lysates were measured on Western blots, a significant increase in TM levels was seen in HAECs exposed to 5, 10, 20, or 40 µM atorvastatin (Fig. 2B). When HAECs were incubated for 2, 6, 12, 24, 48, or 72 h with 10 ng/mL of TNFα, TM expression was, respectively, 70±14%, 59±12%, 46±12%, 35±7%, 31±11%, or 19±3% that in control cells, the reduction for the five longest incubation times being significant (Fig. 2C). When HAECs were incubated for 24 h with various concentrations of TNFα, a significant increase in TM was seen in HAECs exposed to 5, 10, or 20 ng/mL TNFα (Fig. 2D). Toxicity of atorvastatin or TNFα treatment for HAECs was assessed using the MTT assay as shown in Figs. 2E-2H. The incubated time or the incubated concentration, either with atorvastatin or with TNFα as indicated in the figures, did not affect cell viability. When HAECs were pretreated for 24 h with various concentrations of atorvastatin before incubation for 24 h with 10 ng/mL of TNFα, atorvastatin increased TM levels in a dose-dependent manner (Fig. 3A). A significant increase in TM was seen in HAECs exposed to 10 µM or 20 µM atorvastatin. Because the increase in TM levels in response to 10 µM atorvastatin was time-dependent, up to 24 h without evidence of cellular

damage by MTT assay, subsequent experiments were performed using 10 µM atorvastatin for 24 h.

To determine whether TNFα or atorvastatin affected TM mRNA levels, quantitative real-time PCR was performed. Unstimulated HAECs produced low amounts of TM mRNA, and 4 h treatment with 10 ng/mL of TNFα resulted in a marked decrease in levels (Fig. 3B). This decrease was markedly inhibited by preincubation with 10 µM atorvastatin for 24 h. Furthermore, the addition of 10 µg/mL of actinomycin D (an RNA polymerase inhibitor) for 3 h or 5 h significantly reduced TM expression in HAECs treated with atorvastatin in either the presence or absence of TNFα, showing that atorvastatin-induced TM expression required de nova RNA synthesis. Confocal microscopic images showed that TM was distributed in the cytosol in control cells, but not in TNFα-treated HAECs (Fig. 3C). TM expression was stronger in atorvastatin–treated HAECs with or without TNFα stimulation (Fig. 3C). The functional property of TM in HAECs was also assessed by the cell-based measurement of protein C activation. TM activity of control cells showed 1.8-fold higher than that of TNFα-treated HAECs (Fig. 3D). Atorvastatin regulated TM activity with or without TNF· administration and caused a 2.3-fold and 1.5-fold compared with TNFα-treated cells and control cells, respectively. The above results showed that incubation of HAECs for 24 h with atorvastain revealed a prominent increase in cellular TM mRNA and protein, as well as cell surface TM activity.

Atorvastatin inhibits monocyte adhesion to control and TNFα-treated HAECs via TM

To explore the effects of atorvastatin on the HAEC-monocyte interaction, we examined the adhesion of monocytes to TNFα-treated HAECs. As shown in Fig. 4A, control confluent HAECs showed minimal binding of monocytes, but adhesion was substantially increased when the HAECs were treated for 24 h with 10 ng/mL of TNFα. Pretreatment with 10 µM atorvastatin for 24 h significantly reduced the binding of monocytes to HAECs, with or without TNFα stimulation, and increased TM expression. The involvement of TM in adhesion of monocytes to TNFα-treated atorvastatin-treated or nontreated HAECs was therefore examined by pretreatment of the cells with antibody against TM. After HAECs were pretreated with 4 µg/mL of anti-TM antibody for 24 h then incubated with or without atorvastatin, the binding of monocytes to HAECs was 2- or 3.5-fold higher than in atorvastatin-treated and control cells, respectively. The result showed that anti-TM antibody increased the adhesion of monocytes to TNFα-treated HAECs.

Atorvastatin-upregulated TM Expression in control and TNFα-treated HAECs involves the decrease of ERK phosphorylation

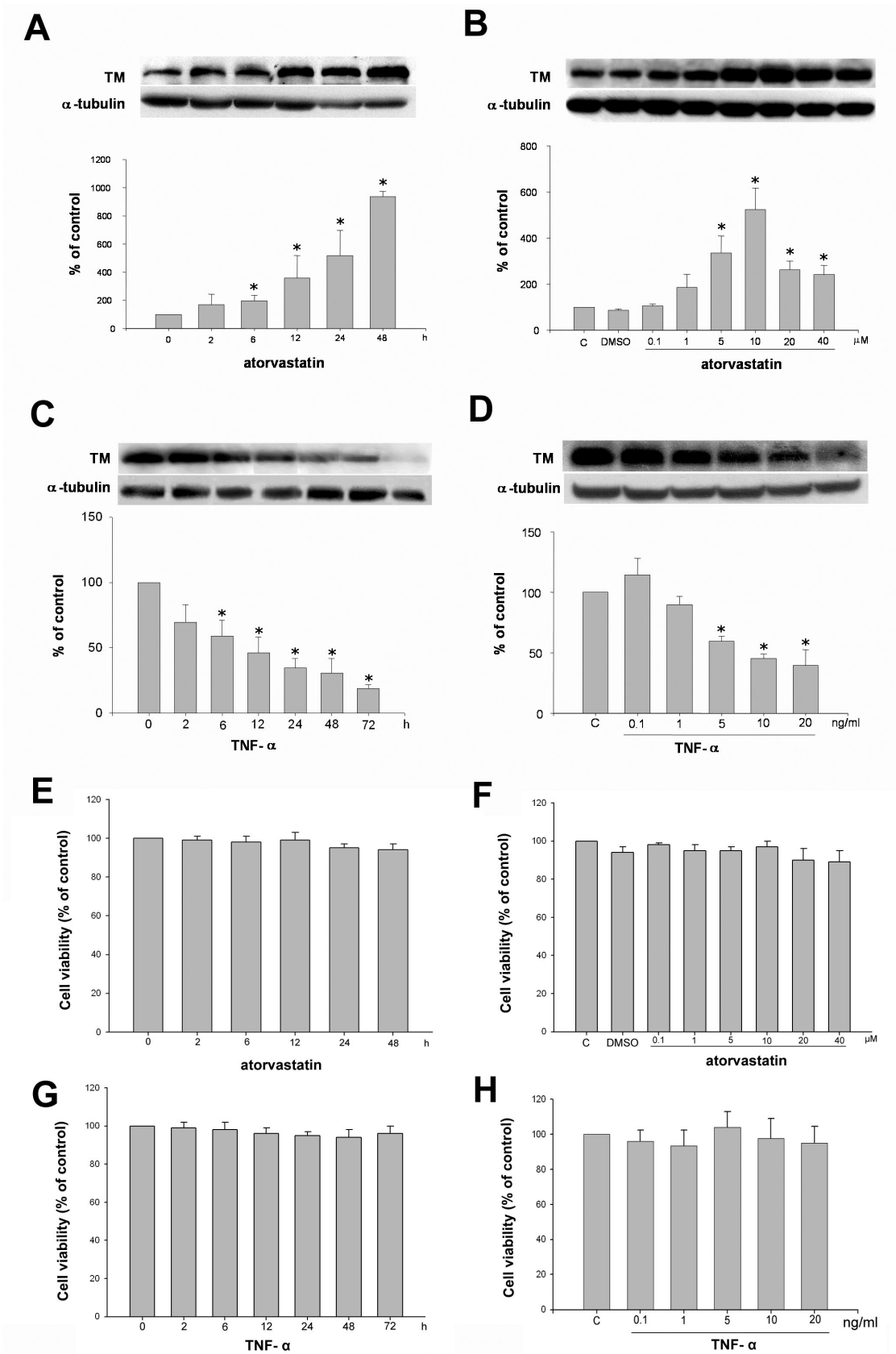Previous studies have shown that TNFα can activate

Fig. 2. In control HAECs, atorvastatin upregulates TM expression, whereas TNFα downregulates TM expression in a time- and dose-dependent manner. **A.** HAECs were incubated for 0-48 h with 10 μM of atorvastatin. **B.** HAECs were incubated for 24 h with various concentrations of atorvastatin. **C.** HAECs were incubated for 0-72 h with 10 ng/mL of TNFα. **D.** HAECs were incubated for 24 h with various concentrations of TNFα. After incubation, TM expression in cell lysates was measured by Western blotting. α-tubulin was used as the loading control. **E-H.** Cytotoxicity of the dose of atorvastatin or TNFα for HAECs was assessed using the MTT assay. The condition of the Figures from E to H was the same as the Figures A to D, respectively. Data are expressed as a percentage of the control value and shown as the mean±SEM for five separate experiments. *P<0.05 compared to untreated cells.

MAPKs in the signaling pathways leading to cytokine production (Baud and Karin, 2001). In the next set of experiments, we examined whether the effects of atorvastatin on TM expression in control cells and TNFα-treated cells occurred via the ERK1/2, p38, or JNK MAPK pathway. As shown in Fig. 5A-C, phosphorylation of ERK1/2, p38, and JNK was significantly increased 20 min after addition of 10 ng/mL of TNFα, but only TNFα-induced ERK1/2 phosphorylation was significantly reduced by 24 h

pretreatment with 10 μM atorvastatin. As shown in Fig. 5D, the decrease in TM expression in response to TNFα treatment was also affected by 1 h pretreatment with 30 μM PD98059 (an ERK1/2 inhibitor).

Atorvastatin Attenuates Activation of NF-κB Expression and Nuclear Translocation of NF-κB p65 in Control and TNFα treated HAECs

A recent study showed that statins reduce the
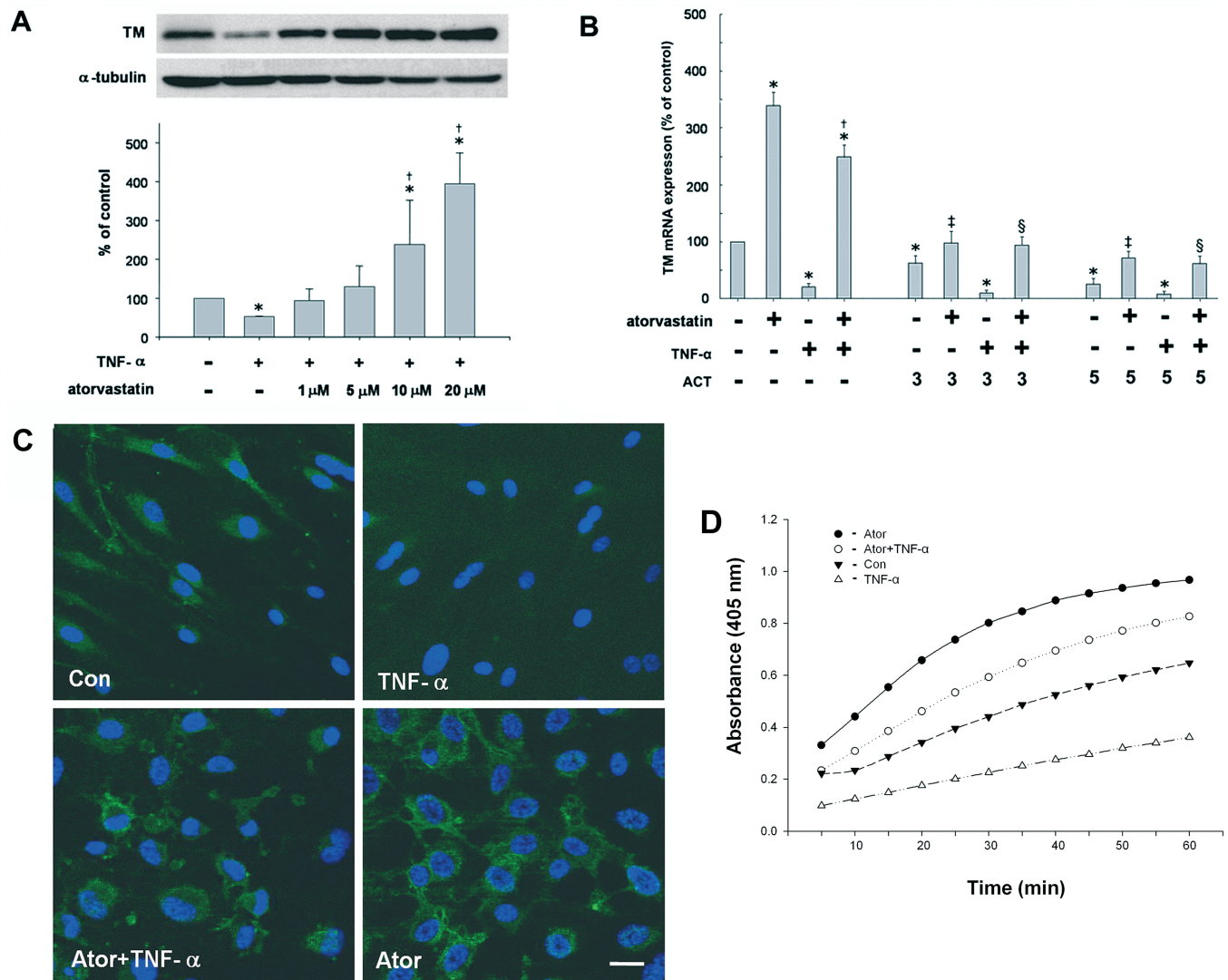
Fig. 3. Atorvastatin upregulates TM mRNA and protein expression in control and TNFα-treated HAECs. **A.** HAECs were incubated for 24 h with various concentrations of atorvastatin, then the cells were incubated with 10 ng/mL of TNFα for 24 h and TM expression measured in cell lysates by Western blotting. α-tubulin was used as the loading control. **B.** HAECs were incubated for 24 h with 10 μM atorvastatin, then the cells were incubated with 10 ng/mL of TNFα for 6 h before TM mRNA expression was analyzed by quantitative real-time PCR after normalization to ß actin mRNA. **C.** The cells were treated as in (A), then the distribution of TM was analyzed by immunofluorescent staining and confocal microscopy. TM expression is indicated by green fluorescence (FITC) and nuclei by blue fluorescence (DAPI). Bar: 40 μm. **D.** TM activity assay. Confluent monolayers of HAECs were assayed for TM-dependent protein C activation activity as described under "Experimental Procedures". The change in absorbance at 405 nm was monitored. In A and B, the 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 cells. †P<0.05 compared to TNFα-treated cells. ‡P<0.05 compared to atorvastatin-treated cells. §P<0.05 compared to atorvastatin+TNFα-treated cells.
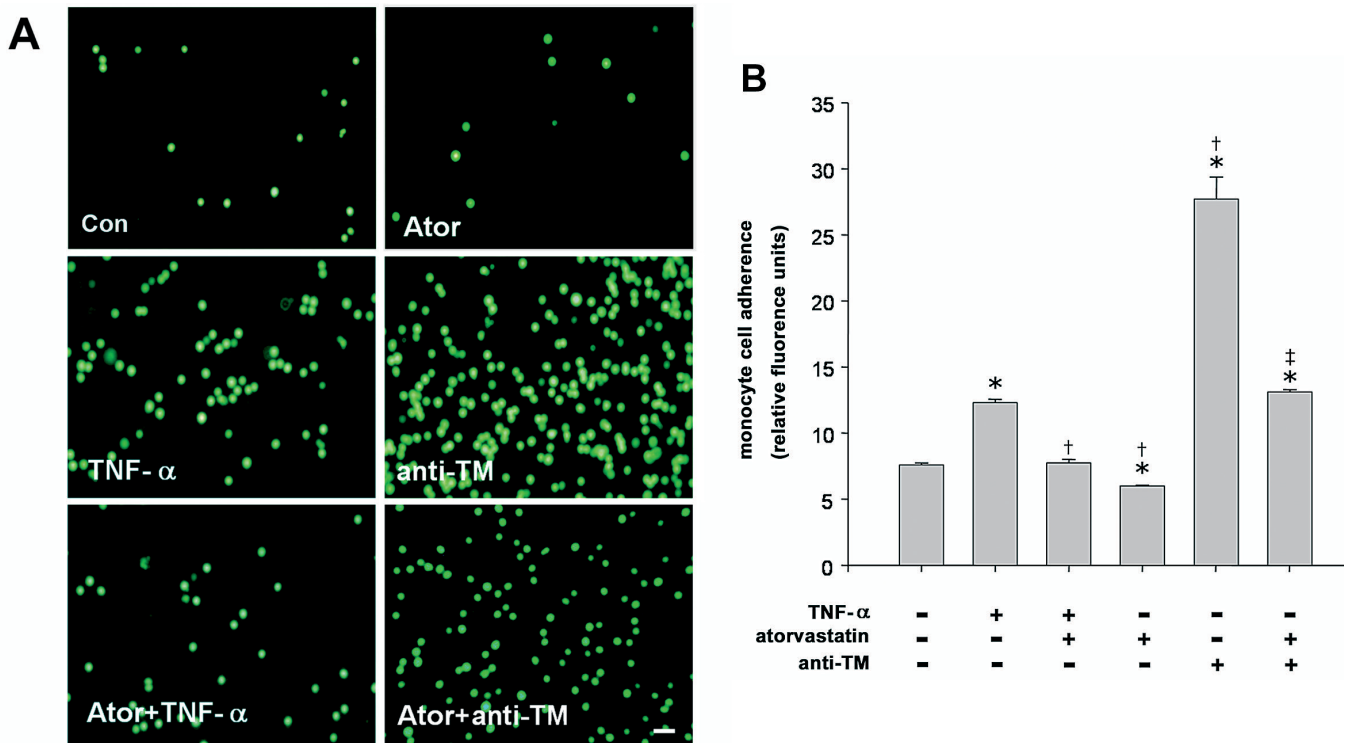
Fig. 4. Atorvastatin reduces the adhesion of U937 cells to control and TNFα-stimulated HAECs. **A.** Representative fluorescent photomicrographs showing the effect of 24 h pretreatment with 10 μM atorvastatin on the TNFα-stimulated (10 ng/mL of TNFα for 24 h) adhesion of fluorescein-labeled U937 cells to HAECs. Bar: 10 μm **B.** Mononuclear cell adherence shown as relative fluorescent units. Pretreatment with atorvastatin significantly reduced the binding of U937 cells to control and TNFα-stimulated HAECs. Pretreatment for 24 h with 4 μg/mL of anti-TM antibody with or without atorvastatin treatment increased binding. *$P<0.05$ compared to untreated cells. †$P<0.05$ compared to TNFα-treated cells. ‡$P<0.05$ compared to atorvastatin-treated cells.

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 atorvastatin on NF-κB activation in TNFα-treated HAECs. As shown in Fig. 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 atorvastatin-pretreated HAECs, the TNFα-induced increase in NF-κB binding was reduced by 90%. As shown by 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). TNFα-treated HAECs showed marked NF-κB p65 staining in the nuclei, while atorvastatin-pretreated cells showed weaker nuclear NF-κB expression, but stronger staining in the cytoplasm (Fig. 6C).

Discussion

In this study, we showed that atorvastatin significantly reduced the intimal area and increased TM

expression in the aorta of cholesterol-fed rabbits. Atorvastatin increased TM mRNA and protein expression in HAECs either with or without TNFα treatment. TNFα significantly increased the phosphorylation of the three MAPKs p38, JNK, and ERK1/2, but only TNFα-induced ERK1/2 phosphorylation was significantly reduced by pretreatment with atorvastatin. Atorvastatin-induced upregulation of TM expression was also mediated by inhibition of NF-κB activation in control and TNFα-treated HAECs.

Epidemiological studies have shown that lipid lowering therapy with statins leads to a significant reduction in cardiac mortality and morbidity. In agreement with a previous report (Aragoncillo et al., 2000), serum cholesterol levels and the area of atherosclerotic lesions were significantly decreased in atorvastatin-treated cholesterol-fed rabbits compared to cholesterol-fed rabbits. In contrast, one study reported that atorvastatin administration for 8 weeks reduces plasma total cholesterol and the size of the iliac-femoral lesion, but thoracic aortic lesions are unchanged (Bocan et al., 1994). A possible explanation for this discrepancy could be differences in the duration of the cholesterol-fed diet and atorvastatin treatment. Atorvastatin also

attenuates tissue factor expression in the adipose tissues of cholesterol-fed rabbits and this is associated with the suppression of procoagulant activity in atherosclerosis (Li et al., 2007). The present study is the first to show increased expression of TM in aortas from atorvastatin-treated rabbits by immunofluorescent staining and Western blotting. The antiatherosclerotic property of atorvastatin may be due to two major factors, one being its pleiotrophic effect, such as improvement of endothelial function, and the other its lipid lowering effect.

To examine how atorvastatin affects TM expression in atherosclerosis, a chronic inflammatory disease, we used HAECs as the cell model and TNFα as the stimulator. Our data showed that TNFα significantly reduced TM expression in HAECs. Because 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 cardiovascular disorders. The vasculoprotective properties of statins have been assumed to result from their ability to upregulate the expression of tissue plasminogen activator and downregulate the expression of tissue factor, plasminogen activator inhibitor-I, and adhesion molecules in stimulated endothelial cells (Morikawa et al., 2002). A microarray study also 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 TM expression (Masamura et al., 2003). Here, we showed that atorvastatin significantly upregulated TM mRNA and protein levels in endothelial cells with or without TNFα treatment. In addition, atorvastatin

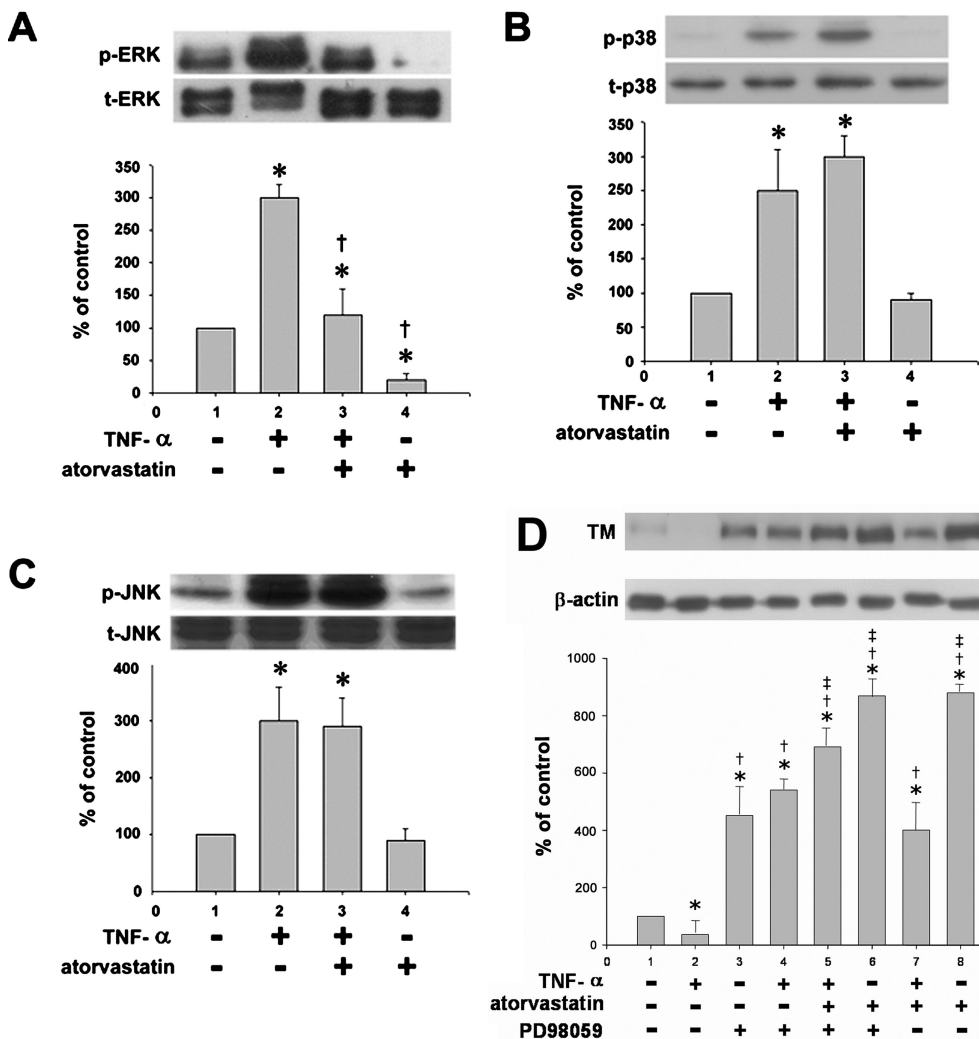**Fig. 5.** Atorvastatin-induced up-regulation of TM expression in control and TNFα-treated HAECs is dependent on the decrease in ERK phosphorylation. **A-C.** Western blot analysis showing the effect of atorvastatin pretreatment on the phosphorylation of **(A)** ERK1/2, **(B)** p38, or **(C)** JNK in TNFα-treated and control HAECs. HAECs were incubated for 24 h with or without 10 μM atorvastatin, then the cells were 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 the indicated antibodies. **D.** Effect of inhibitors of MAPK phosphorylation on TM expression in control and TNFα-treated HAECs. HAECs were incubated for 23 h with atorvastatin (10 μM) and for 1 h with medium or 30 μM PD98059 (an ERK1/2 inhibitor) in the presence of atorvastatin, then the cells were 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 3 separate experiments. Total ERK (t-ERK), total p38 (t-p38), total JNK (t-JNK), or ß-actin was used as the loading control for Fig 5A, 5B, 5 C, 5D, respectively. *P<0.05 compared to untreated cells. †P<0.05 compared to TNFα-treated cells. ‡P<0.05 compared to atorvastatin +TNFα-treated cells.

A

TNF-α	-	+	-	+
atorvastatin	-	-	+	+

NF-κB →

free probe →

B

TM

α-tubulin

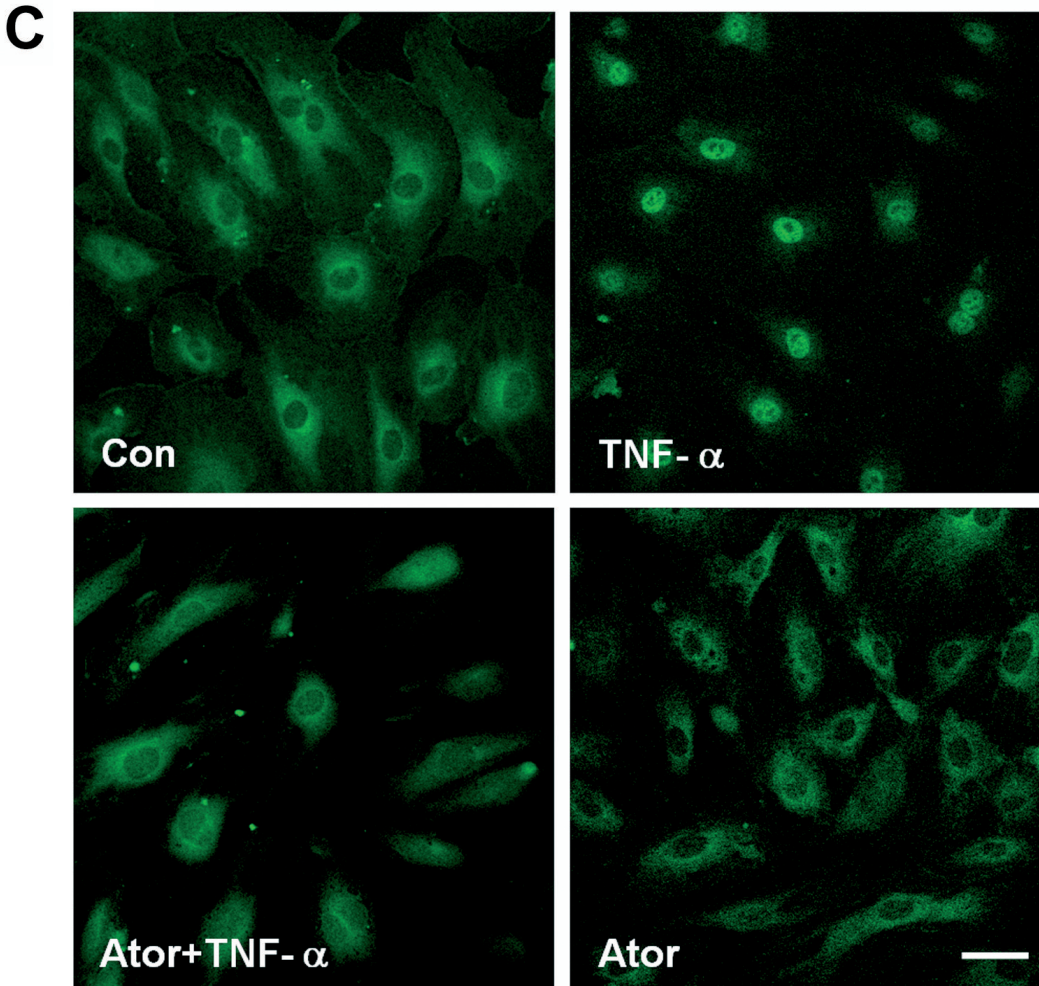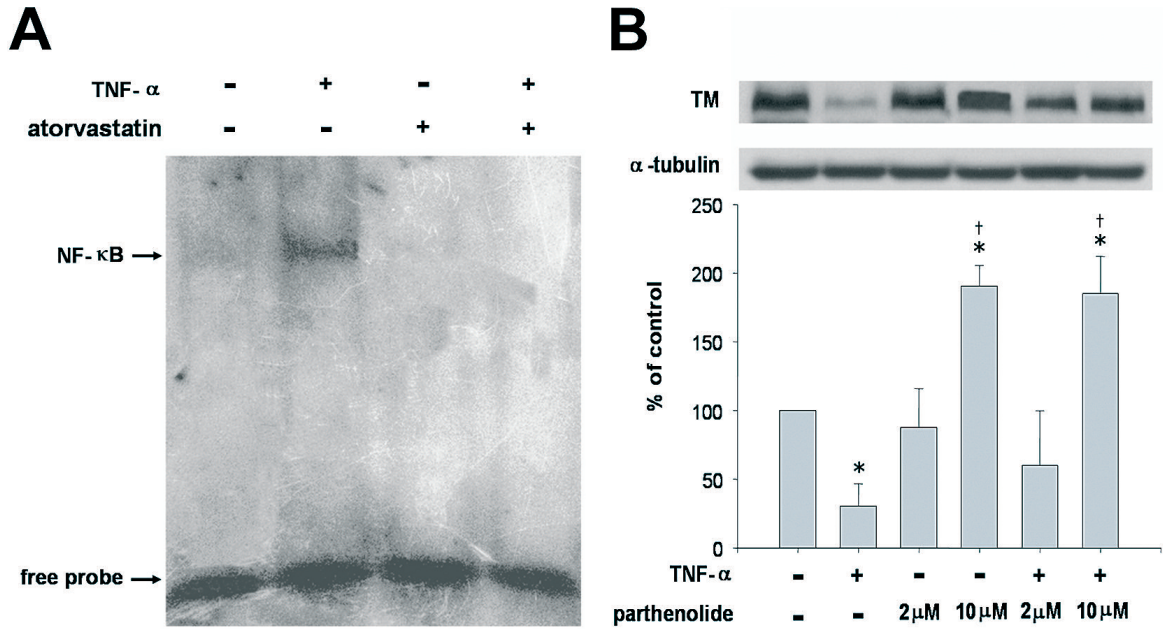|						
TNF-α	-	+	-	-	+	+
parthenolide	-	-	2μM	10μM	2μM	10μM

y-axis: % of control (0–250)

C

Con

TNF-α

Ator+TNF-α

Ator

Fig. 6. Atorvastatin-induced upregulation of TM expression in TNFα-stimulated HAECs is mediated by inhibition of both NF-κB activation and NF-κB p65 nuclear translocation. **A.** Nuclear extracts prepared from untreated cells or from cells with or without atorvastatin pretreatment (10 μM, 24 h) subsequently incubated with 10 ng/mL of TNFα for 30 min were 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 cell lysates were prepared and assayed for TM on Western blots. **C.** Immunofluorescence staining for NF-κB p65. Higher expression of NF-κB p65 protein is seen in the nuclei of TNFα-stimulated HAECs compared to control HAECs and atorvastatin pretreatment reduces NF-κB p65 expression. A representative result from three separate experiments is shown and the summarized data for the three experiments are shown in the bar chart. Bar: 40μm. *P<0.05 compared to untreated cells, †P<0.05 compared to TNFα-treated cells.

increased TM transcriptional levels by de nova RNA synthesis and did not extend the stability of TM mRNA, as shown using actinomycin D.

HAECs treated with 10 µM atorvastatin for 24 h increased TM protein level about 5-fold compared to untreated control cells in the present study. Our result was similar to those of other studies. Atorvastatin increased TM mRNA levels more than 10-fold and resulted in strong enhancement of protein C activation in EA. hy 926 endothelial cells (Shi et al., 2003). Levels of TM antigen and mRNA showed a marked concentration- and time-dependent increase after treatment with pitavastatin in human umbilical endothelial cells (Masamura et al., 2003). For pitavastatin at the concentration of 10^{-5} M for 24 h, TM mRNA levels are 3 times as high as the control levels. Atorvastatin increased TM mRNA level to about 4.5 times the control levels in human umbilical vein endothelial cells (Fu et al., 2008). The main feature of the antithrombotic properties of normal endothelial cells is caused by the constitutive expression of TM. The increased TM expression of normal HAECs under atorvastatin treatment may provide stronger protection against external stress.

Statins may inhibit vascular inflammation and the development of atherosclerosis by interfering with leukocyte trafficking. Simvastatin decreases neutrophil infiltration into myocardial infarcts by inhibiting selectin externalization and decreases the extent of myocardial infarcts in mice (Yamakuchi et al., 2005). The present study showed that the atorvastatin-induced decrease in monocyte-EC adhesion with or without TNFα stimulation was mediated by upregulation of TM. Because of the involvement of TM in monocyte recruitment to early atherosclerotic lesions, our findings suggest an additional mechanism by which atorvastatin may be involved in preventing the progression of atherosclerosis. A mouse monoclonal TM antibody treatment increased the adhesion of monocytes to TNFα-treated HAECs. The antibody used in the present study raised against amino acids 22-321 mapping within the extracellular domain of TM. TM has five domains, including the NH2 domain (lectin-like domain, amino acid residues 1-226), a domain with six epidermal growth factor-like structures (aa residues 227-462), an O-glycosylation site-rich domain (aa residues 463-497), a 24-residue transmembrane domain (aa residues 498-521) and a cytoplasmic domain (Suzuki et al., 1987). The lectin-like domain of TM suppressed polymorphonuclear leukocyte (PMN) adhesion to ECs by reducing adhesion molecule expression (Conway et al., 2002). Another likely candidate region would be the first two EGF-like repeats, these structures being classically involved in protein–protein interactions. These EGF-like repeats could provide a receptor site for activated PMNs that are unmasked during inflammation by allosteric changes in the NH_2-terminal domain or by its cleavage with proteases released from monocytes. The anti-TM antibody increased monocyte adhesion to endothelial cells in the present study could be explained in part by steric interference with the proadhesive effect of the EGF-like repeats. Additional studies are necessary to explore the effects of TM on monocytes adhesion to ECs under statin treatment.

In general, the pleiotropic effects of statins are thought to be mediated by their ability to affect MAPK phosphorylation (Tristano and Fuller, 2006). Atorvastatin-induced VEGF release in cardiac myocytes is positively regulated by p38 MAPK and negatively regulated by ERK (Nakajima et al., 2006). Simvastatin treatment does not modulate TNFα-induced ERK and p38 phosphorylation in human cardiac myofibroblasts (Turner et al., 2007). Pravastatin upregulates TM expression in TNFα-treated HAECs is not associated with MAPK phosphorylation (Lin et al., 2007). In the present study we showed that atorvastatin pretreatment caused a significant reduction in TNFα-induced phosphorylation of ERK. In addition, an ERK inhibitor (PD98059) had a significant effect on TM expression in normal or TNFα-treated HAECs. This study showed that the atorvastatin-induced upregulation of TM expression in control and TNFα-treated cells involves ERK and not JNK and p38. The differences in the types of MAPKs phosphorylated by statin treatment in different studies may be due to differences in the cell types, cytokines, inducers, and kinds of statins used.

The major transcription factor, NF-κB, has an important function in the regulation of many genes involved in chronic and acute inflammatory responses (Dichtl et al., 2003), and recent studies indicate that it is involved in the pathogenesis of atherosclerosis (de Winther et al., 2005). Lipopolysaccharide downregulates TM expression in monocytes by blocking NF-κB activation (Kim et al., 2007). In the present study, we showed that atorvastatin prevented the increase in NF-κB activity caused by TNFα. Parthenolide, which blocks NF-κB activation, prevented the TNFα-induced downregulation of TM expression. These results suggest that NF-κB acts 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 coactivator, p300, which is required for TM gene expression (Sohn et al., 2005). Krüppel-like factor (KLF2), another transcription factor, has been demonstrated to be important in the statin-induced expression of eNOS and TM (Sen-Banerjee et al., 2005). It is possible that some of the inhibitory effects of NF-κB on TM expression may be secondary to an increase in KLF2 levels. We will investigate this in future studies.

In summary, atorvastatin potently increased TM expression in HAECs and counteracted the TNFα-induced downregulation of TM. This effect was mediated, at least in part, by the ERK/NF-κB signaling pathways. In addition, atorvastatin treatment reduced the

intimal area in the thoracic aortas and induced significant TM expression in the intima in cholesterol-fed rabbits. These findings demonstrate an important mechanism by which atorvastatin enhances the anticoagulant and anti-inflammatory properties of the vascular endothelium. These effects of statins may be particularly important in patients with acute coronary syndromes.

References

Aragoncillo P., Maeso R., Vazquez-Perez S., Navarro-Cid J., Ruilope L.M., Diaz C., Hernandez G., Lahera V. and Cachofeiro V. (2000). The protective role of atorvastatin on function, structure and ultrastructure in the aorta of dyslipidemic rabbits. Virchows Arch. 437, 545-554.

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

Bocan T.M.A., Mazur M.J., Mueller S.B., Brown E.Q., Sliskovic D.R., O'Brien P.M., Creswell M.W., Lee H., Uhlendorf P.D., Roth B.D. and Newton R.S. (1994). Antiatherosclerotic activity of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase in cholesterol-fed rabbits: a biochemical and morphological evaluation. Atherosclerosis 111, 127-142.

Calabro P. and Yeh E.T.H. (2005). The pleiotropic effects of statins. Curr. Opin. Cardiol. 20, 541-546.

Chen Y.H., Lin S.J., Chen J.W., Ku H.H. and Chen Y.L. (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 E.M., Van de Wouwer M., Pollefeyt S., Jurk K., Van Aken H., De Vriese A., Weitz J.I., Weiler H., Hellings P.W., Schaeffer P., Herbert J.M., Collen D. and Theilmeier G. (2002). The lectin-like domain of thrombomodulin confers protection from neutrophil-mediated tissue damage by suppressing adhesion molecule expression via nuclear factor kappaB and mitogen-activated protein kinase pathways. J. Exp. Med. 196, 565-577.

de Winther M.P.J., Kanters E., Kraal G. and Hofker M.H. (2005). Nuclear factor kappaB signaling in atherogenesis. Arterioscler. Thromb. Vasc. Biol. 25, 904-914.

Devaraj S., Chan E. and Jialal I. (2006). Direct demonstration of an antiinflammatory effect of simvastatin in subjects with the metabolic syndrome. J. Clin. Endocrinol. Metab. 91, 4489-4496.

Dichtl W., Dulak J., Frick M., Alber H.F., Schwarzacher S.P., Ares M.P.S., Nilsson J., Pachinger O. and 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.

Esmon C.T. (2003). Inflammation and thrombosis. J. Thromb. Haemost. 1, 1343-1348.

Fu Q., Wang J., Boerma M., Berbée M., Qiu X., Fink L.M. and Hauer-

Jensen M. (2008). Involvement of heat shock factor 1 in statin-induced transcriptional upregulation of endothelial thrombomodulin. Circ Res. 103,369-377.

Kim H.K., Kim J.E., Chung J., Kim Y.T., Kang S.H., Han K.S. and Cho H.I. (2007). Lipopolysaccharide down-regulates the thrombomodulin expression of peripheral blood monocytes: effect of serum on thrombomodulin expression in the THP-1 monocytic cell line. Blood Coagul. Fibrin. 18, 157-164.

Li J.Q., Zhao S.P., Li Q.Z., Cai Y.C., Wu L.R., Fang Y. and Li P. (2007). Atorvastatin reduces tissue factor expression in adipose tissue of atherosclerotic rabbits. Int. J. Cardiol. 115, 229-234.

Lin S.J., Chen Y.H., Lin F.Y., Hsieh L.Y., Wang S.H., Lin C.Y., Wang Y.C., Ku H.H., Chen J.W. and Chen Y.L. (2007). Pravastatin induces thrombomodulin expression in TNFalpha-treated human aortic endothelial cells by inhibiting Rac1 and Cdc42 translocation and activity. J. Cell Biochem. 101, 642-653.

Masamura K., Oida K., Kanehara H., Suzuki J., Horie S., Ishii H. and 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.

Medina P., Navarro S., Estelles A. and Espana F. (2007). Polymorphisms in the endothelial protein C receptor gene and thrombophilia. Thromb. Haemost. 98, 564-569.

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

Nakajima K., Suga H., Matsuno H., Ishisaki A., Hirade K. and Kozawa O. (2006). Differential roles of MAP kinases in atorvastatin-induced VEGF release in cardiac myocytes. Life Sci. 79, 1214-1220.

Rosenson R.S. and Tangney C.C. (1998). Antiatherothrombotic properties of statins: implications for cardiovascular event reduction. JAMA 279, 1643-1650.

Ross R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362, 801-809.

Seljeflot I., Tonstad S., Hjermann I. and Arnesen H. (2002). Reduced expression of endothelial cell markers after 1 year treatment with simvastatin and atorvastatin in patients with coronary heart disease. Atherosclerosis 162, 179-185.

Sen-Banerjee S., Mir S., Lin Z., Hamik A., Atkins G.B., Das H., Banerjee P., Kumar A.and Jain M.K. (2005). Krüppel-like factor 2 as a novel mediator of statin effects in endothelial cells. Circulation 112, 720-726.

Shepherd J., Cobbe S.M., Ford I., Isles C.G., Lorimer A.R., MacFarlane P.W., McKillop J.H. and Packard C.J. (1995). Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. N. Engl. J. Med. 333, 1301-1307.

Shi J., Wang J., Zheng H., Ling W., Joseph J., Li D., Mehta J.L., Ponnappan U., Lin P., Fink L.M. and 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 14,575-85.

Sohn R.H., Deming C.B., Johns D.C., Champion H.C., Bian C., Gardner K. and Rade J.J. (2005). Regulation of endothelial thrombomodulin expression by inflammatory cytokines is mediated by activation of nuclear factor-kappa B. Blood 105, 3910-3917.

Suzuki K., Kusumoto H., Deyashiki Y., Nishioka J., Maruyama I., Zushi

M., Kawahara S., Honda G., Yamamoto S. and Horiguchi S. (1987). Structure and expression of human thrombomodulin, a thrombin receptor on endothelium acting as a cofactor for protein C activation. EMBO J. 6, 1891-1897.

Tristano A.G. and Fuller K. (2006). Immunomodulatory effects of statins and autoimmune rheumatic diseases: novel intracellular mechanism involved. Int. Immunopharmacol. 6, 1833-1846.

Turner N.A., Aley P.K., Hall K.T., Warburton P., Galloway S., Midgley L., O'Regan D.J., Wood I.C., Ball S.G. and Porter K.E. (2007). Simvastatin inhibits TNFalpha-induced invasion of human cardiac myofibroblasts via both MMP-9-dependent and -independent mechanisms. J. Mol. Cell Cardiol. 43, 168-176.

Undas A., Brummel-Ziedins K.E. and Mann K.G. (2005). Statins and blood coagulation. Arterioscler. Thromb. Vasc. Biol. 25, 287-294.

Yamakuchi M., Greer J.J.M., Cameron S.J., Matsushita K., Morrell C.N., Talbot-Fox K., Baldwin W.M., Lefer D.J. and Lowenstein C.J. (2005). HMG-CoA reductase inhibitors inhibit endothelial exocytosis and decrease myocardial infarct size. Circ. Res. 96, 1185-1192.