Statins Prevent Tissue Factor Expression in Human Endothelial Cells
Role of Rho/Rho-Kinase and Akt Pathways
Masato Eto, MD; Toshiyuki Kozai, MD; Francesco Cosentino, MD, PhD; Hana Joch; Thomas F. Lüscher, MD, FRCP

Background—Tissue factor plays a pivotal role in thrombus formation in acute coronary syndromes. However, the regulatory mechanisms underlying tissue factor expression are poorly understood. Statins are effective in patients with acute coronary syndromes. Hence, the aim of this study was to clarify in human endothelial cells the signaling pathways of thrombin-induced tissue factor expression and potential inhibitory effects of statins.

Methods and Results—In human aortic endothelial cells, simvastatin prevented tissue factor induction by thrombin (4 U/mL) in a concentration-dependent manner. The increase in tissue factor activity on the cell surface was also blocked by simvastatin. Simvastatin also prevented the upregulation of tissue factor expression and activity in human aortic smooth muscle cells. Mevalonate (100 μmol/L) reversed the inhibitory effect of simvastatin on tissue factor expression. Thrombin induced rapid activation of Rho A and p38 MAP kinase. The Rho-kinase inhibitor Y-27632 and the p38 MAP kinase inhibitor SB203580 prevented tissue factor induction. Akt was dephosphorylated by thrombin; the phosphoinositide 3-kinase inhibitor wortmannin enhanced its dephosphorylation as well as thrombin-induced tissue factor expression. Simvastatin prevented thrombin-induced Rho A activation but not p38 MAP kinase activation. Akt dephosphorylation by thrombin was blocked by both simvastatin and Y-27632.

Conclusions—Endothelial tissue factor induction by thrombin is regulated by Rho/Rho-kinase, Akt, and p38 MAP kinase. Simvastatin prevents its induction through inhibition of Rho/Rho-kinase and activation of Akt. These findings provide new insights into the action of statins in acute coronary syndromes. (Circulation. 2002;105:1756-1759.)

Key Words: endothelium ▪ pharmacology ▪ signal transduction ▪ thrombosis

Acivation of the coagulation cascade plays a pivotal role in the pathogenesis of thrombotic vascular disease, in particular acute coronary syndromes.1–4 This cascade is initiated by binding of tissue factor to activated factor VII, and this complex activates factor X. Activated factor X generates thrombin and in turn thrombin cleaves fibrinogen to fibrin. Thrombin also activates cell-surface receptors in several types of cells.5 Indeed, thrombin induces platelet aggregation6 and tissue factor expression.7 Thus, thrombin is a key modulator of this positive feedback mechanism of thrombus formation. However, the signaling pathways responsible for thrombin-induced tissue factor expression remain unclear.

Statins are widely used in clinical practice because they are effective in the prevention of cardiovascular events.8 Most likely, these beneficial effects are due to not only an improved lipid profile but also direct vascular actions.9 Statins inhibit HMG-CoA reductase, which is a rate-limiting enzyme of the mevalonate-cholesterol pathway. Activation of this pathway leads to the production of intermediates such as all-trans geranylgeranyl pyrophosphate. This intermediate activates Rho by posttranslational modification, a process of which is inhibited by statins.10

It was the aim of this study to investigate the signaling mechanisms underlying thrombin-induced tissue factor expression in human aortic endothelial cells (HAECs) and the potential preventive effects of statins on tissue factor induction.

Methods
Thrombin was purchased from Sigma. Y-27632, wortmannin, SB203580, and U0126 were purchased from Calbiochem. Simvastatin was kindly provided by MSD.
Cell Culture
HAECs and human aortic smooth muscle cells (HASMCs) were obtained from Clonetics. In 60-mm culture dishes, HAECs and HASMCs were grown in endothelial basal medium (Clonetics) and DMEM (Gibco), respectively, in a humidified atmosphere (37°C, 95% air/5% CO₂). Both media were supplemented with 10% FCS, 20 mmol/L L-glutamine, 10 mmol/L HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin. After confluence, cells were rendered quiescent by incubation in medium with 0.5% serum for 24 hours and then stimulated with thrombin. HAECs of 4th to 8th passages and HASMCs of 5th to 10th passages were used in experiments.

Tissue Factor Expression
Tissue factor expression was determined with Western blotting. The samples (30 μg) were treated with SDS-PAGE sample buffer, followed by heating and then subjected to 10% gel. The protein was transferred onto membranes with a semidry transfer unit. Western blotting was performed with tissue factor antibody (1:100, Calbiochem). The bands were detected by a chemiluminescent system. Restaining with tubulin antibody (1:1000, Sigma) ensured equal loading.

Tissue Factor Activity
Tissue factor activity was determined with the use of an Actichrome Kit (American Diagnostica). After 6-hour stimulation with thrombin, cells were incubated with human factor VIIa (10 nmol/L, American Diagnostica) and substrate Spectroenzyme fVIIa (500 μmol/L, American Diagnostica) for 30 minutes in phenol red–free DMEM at 37°C. Then medium was removed, and optical density was monitored spectrophotometrically at 405 nm. Recombinant tissue factor (American Diagnostica) was used for calibration.

MAP Kinases and Akt Phosphorylation
Phosphorylation of ERK, p38 MAP kinase, and Akt was determined with the use of phospho-specific antibodies (Cell Signaling, Allschwil, Switzerland). The protein (30 μg) was electrophoresed in 12% SDS-PAGE gel. Western blotting was performed with the use of phospho-ERK (1:1000), phospho-p38 (1:200), or phospho-Akt (1:100) antibodies.

Rho A Activity
Rho A activity was determined by a pull-down assay. The cell lysates were incubated with Rhotekin Rho Binding Domain (Upstate Biotechnology, Lake Placid, NY) for 45 minutes. The agarose beads were collected and electrophoresed in 12% SDS-PAGE gel. Western blotting was performed with RhoA antibody (1:1000, Upstate Biotechnology).

Statistics
Data are given as mean±SEM. Statistical analysis was performed with an unpaired t test between 2 groups and ANOVA among more than 3 groups. A value of P < 0.05 was considered to indicate statistical difference.

Figure 1. Simvastatin prevents thrombin-induced upregulation of tissue factor expression and activity in HAECs. A, Effect of simvastatin on tissue factor induction by thrombin, determined by Western blotting. Left panel shows the representative blots; right panel shows quantitative analysis with densitometry (n=3). B, Effect of mevalonate on inhibitory action of simvastatin on tissue factor induction. Left panel shows representative blots; right panel shows quantitative analysis with densitometry (n=3). C, Effect of simvastatin on increase in tissue factor activity induced by thrombin on cell surface (n=3).
Results

Statin Prevents Thrombin-Induced Tissue Factor Expression and Activity

Thrombin (4 U/mL) markedly increased tissue factor expression with a maximal induction after 4 to 6 hours of incubation (data not shown) in HAECs. Tissue factor induction was concentration-dependent (0.01 to 4 U/mL, data not shown). Simvastatin significantly prevented tissue factor induction by thrombin in a concentration-dependent manner (10 nmol/L to 1 µmol/L, Figure 1A). Mevalonate (100 µmol/L) completely reversed the inhibitory effect of simvastatin (Figure 1B).

Thrombin also increased cell-surface tissue factor activity in HAECs (Figure 1C). Again, simvastatin significantly prevented the increase in tissue factor activity (Figure 1C).

Similar to HAECs, in HASMCs, simvastatin (1 µmol/L) significantly prevented thrombin-induced (4 U/mL, 6 hours) tissue factor expression and increase in tissue factor activity (Supplementary Data).

Role of Rho/Rho-kinase, MAP Kinases, and Akt

Thrombin (4 U/mL) induced rapid (5 to 10 minutes) activation of Rho A, p38 MAP kinase, and ERK1/2 in HAECs (data not shown). The Rho-kinase inhibitor, Y-27632 (10 µmol/L), as
well as the p38 MAP kinase inhibitor, SB203580 (10 μmol/L), significantly prevented tissue factor induction by thrombin (Figure 2A). However, inhibition of the MEK/ERK pathway by the MEK1/2 inhibitor U0126 (10 μmol/L) failed to prevent tissue factor induction (Figure 2A).

In contrast to Rho and MAP kinases, Akt was dephosphorylated by thrombin (Figure 2B). The phosphoinositol 3-kinase inhibitor, wortmannin (100 nmol/L), enhanced thrombin-induced dephosphorylation of Akt, as expected (Figure 2B). Wortmannin significantly potentiated tissue factor induction by thrombin (Figure 2B).

**Effects of Simvastatin on Signaling**

Simvastatin prevented Rho A activation induced by thrombin (Figure 2C). However, simvastatin failed to block p38 MAP kinase activation (Figure 2C). Akt dephosphorylation by thrombin was also inhibited by simvastatin (Figure 2C). Finally, we examined whether Y-27632 could mimic the inhibitory action of simvastatin on Akt dephosphorylation. Similarly, this process was also blocked by Y-27632 (Figure 2C).

**Discussion**

Thrombin acts as a key modulator of thrombus formation through the conversion of fibrinogen to fibrin and the induction of tissue factor. However, the signaling mechanisms underlying tissue factor expression are not fully understood. Here, we showed that thrombin-induced tissue factor expression is regulated positively by Rho/Rho-kinase and p38 MAP kinase. Indeed, both pathways were activated by thrombin, and the specific inhibitors prevented tissue factor expression. Interestingly, we showed for the first time that thrombin dephosphorylates Akt, suggesting that thrombin activates a phosphatase that dephosphorylates and inactivates Akt. The fact that wortmannin enhanced tissue factor expression by thrombin demonstrates that this pathway plays a negative regulatory role in this context.

Most importantly, we observed that simvastatin reduced thrombin-induced upregulation of tissue factor expression in HAECS as well as HASMCs. Tissue factor expression in monocytes/macrophages is inhibited by statins, but thus far, the underlying signaling pathways have not been elucidated. In this study, the reduction of tissue factor expression was reversed by mevalonate, indicating that inhibition of the mevalonate pathway mediates the effect of statin. This pathway is known to generate all-trans geranylgeranyl pyrophosphate as an intermediate, which is essential for Rho activation. Indeed, in this study, Rho activation was blocked by simvastatin. Furthermore, simvastatin inhibited Akt dephosphorylation by thrombin. Statin has been reported to activate Akt; however, the underlying mechanisms were unclear. In this study, Akt dephosphorylation was blocked by Y-27632, suggesting that Rho-kinase negatively regulates Akt. Thus, inhibition of Rho-kinase–dependent Akt dephosphorylation mediates, at least in part, the inhibitory effect of simvastatin on tissue factor.

In this study, the effect of simvastatin was observed from 100 nmol/L to 1 μmol/L. This level is close to peak plasma concentration in humans after an administration of relatively high doses of simvastatin, suggesting that the effects of simvastatin observed in this study are clinically relevant. In addition, recent morphological studies have clearly shown that endothelial cells, smooth muscle cells, and macrophages express tissue factor in human atherosclerotic plaque. Macrophages are believed to be a major source of tissue factor. Tissue factor in endothelial cells and smooth muscle cells, however, is also considered to contribute to plaque thrombogenicity. Although the relative contribution of these three cell types remains unclear, the inhibitory effects of statins on tissue factor expression, shown here in HAECS and HASMCs and by others in macrophages, may explain, at least in part, their beneficial clinical effects on cardiovascular events.

In conclusion, simvastatin prevents endothelial tissue factor induction through inhibition of Rho/Rho-kinase and activation of Akt. These findings provide new insights into the molecular mechanisms of action of statins on vascular wall. These effects of statins may be particularly important in patients with acute coronary syndromes.

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