3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors Increase Fibrinolytic Activity in Rat Aortic Endothelial Cells
Role of Geranylgeranylation and Rho Proteins

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Abstract—3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (HRIs) have been recently shown to prevent atherosclerosis progression. Clinical benefit results from combined actions on various components of the atherosclerotic lesion. This study was designed to identify the effects of HRI on one of these components, the endothelial fibrinolytic system. Aortas isolated from rats treated for 2 days with lovastatin (4 mg/kg body wt per day) showed a 3-fold increase in tissue plasminogen activator (tPA) activity. In a rat aortic endothelial cell line (SVARECs) and in human nontransformed endothelial cells (HUVECs), HRI induced an increase in tPA activity and antigen in a time- and concentration-dependent manner. In SVARECs, the maximal response was observed when cells were incubated for 48 hours with 50 \( \mu \text{mol/L} \) HRI. An increase of tPA mRNA was also in evidence. In contrast, HRI inhibited plasminogen activator inhibitor-1 activity and mRNA. The effects of HRI were reversed by mevalonate and geranylgeranyl pyrophosphate, but not by LDL cholesterol and farnesyl pyrophosphate, and were not induced by \( \alpha \)-hydroxyfarnesyl phosphonic acid, an inhibitor of protein farnesyl transferase. C3 exoenzyme, an inhibitor of the geranylgeranylated-activated Rho protein, reproduced the effect of lovastatin on tPA and plasminogen activator inhibitor-1 activity and blocked its reversal by geranylgeranyl pyrophosphate. The effect of HRI was associated with a disruption of cellular actin filaments without modification of microtubules. A disrupter of actin filaments, cytochalasin D, induced the same effect as lovastatin on tPA, whereas a disrupter of microtubules, nocodazole, did not. In conclusion, HRI can modify the fibrinolytic potential of endothelial cells, likely via inhibition of geranylgeranylated Rho protein and disruption of the actin filaments. The resulting increase of fibrinolytic activity of endothelial cells may contribute to the beneficial effects of HRI in the progression of atherosclerosis. (Circ Res. 1998;83:683-690.)

Key Words: atherosclerosis ■ plasminogen activator ■ isoprenoid ■ Rho protein ■ endothelium

Recent clinical trials have demonstrated that the major function of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (HRIs) is to prevent cardiovascular diseases. HRIs were shown to reduce cardiovascular events in patients suffering from coronary artery diseases, in patients with hypercholesterolemia but no artery disease, and in patients without hypercholesterolemia. HRIs also prevented the progression of atherosclerosis in coronary artery bypass grafts. HRIs were designed to inhibit the endogenous synthesis of cholesterol, thereby decreasing LDL synthesis in liver, thus leading to a reduction of lipid deposition in arteries and to a delayed growth of atherosclerotic plaques. However, some studies raised the question of the exclusive involvement of the decrease in plasma LDL concentration on the effects of HRIs. Indeed, HRIs induced clinical benefits before any regression in atherosclerotic plaques could be detected; they diminished cardiovascular mortality even in normocholesterolemic or hypocholesterolemic patients, and their effects were different from those observed after reduction of plasma cholesterol levels by surgical therapy. All these results suggest that the clinical benefit of HRI administration may result from multiple effects on the components of the atherosclerotic lesions. Atherosclerosis and vascular thrombosis result from complex changes and interactions between blood vessels and blood constituents, of which, fibrin deposition and fibrin lysis are major factors. Fibrinolysis results from the cleavage of fibrin by plasmin, a serine protease that is activated by the plasminogen activators (PAs). Two physiological PAs have been described, tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). In turn, tPA and uPA activity are tightly regulated by a specific inhibitor, plasminogen activator inhibitor-1 (PAI-1).

Abnormalities such as high fibrinogen levels or high PAI-1 and low tPA levels have been described in patients...
with hyperlipoproteinemia. Lipoprotein(a), which reduces tPA-mediated clot lysis,\textsuperscript{11} and PAI-1 have also been shown to be an independent risk factor for myocardial infarction.\textsuperscript{12}

In vivo studies of the effect of HRIs on fibrinolysis, in animals or in humans, led to conflicting results on tPA activity,\textsuperscript{13,14} and these discrepant results may be explained by a rapid clearance of PAs and PA/PAI-1 complexes by receptor-mediated endocytosis in the liver.\textsuperscript{10} Thus, analysis of plasma tPA and/or PAI-1 activity may not reflect the local modification of the fibrinolytic system, which could regulate the atherosclerotic process.

Since the local fibrinolytic activity in blood vessels is determined to a large extent by the endothelial cell production of tPA and PAI-1,\textsuperscript{1,15} we investigated the effect of HRI on the balance between tPA and PAI-1. We show that HRIs induce an increase of tPA synthesis and release and a decrease in PAI-1 activity. The resulting increase in local fibrinolytic activity may explain the beneficial effects observed with HRIs on atherosclerosis progression.

### Materials and Methods

**Materials**

Lovastatin and simvastatin were kindly provided by Merck Sharp & Dohme-Chibret (Rahway, NJ). Mevastatin (compactin), mevalonic acid lactone, LDL cholesterol, farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), cytochalasin D, nocodazole, mouse monoclonal antibody anti-α-tubulin, FITC-conjugated secondary antibody, TRITC-phalloidin, bovine fibrinogen, and bovine thrombin were purchased from Sigma Chemical Co.

A 10% solution of nonfat dried milk in PBS containing 0.1% formamide, 6 \textsuperscript{3} \textsuperscript{2} C\textsubscript{N}

*Supernatants of SVARECs were collected, centrifuged (15 minutes, 14 000g, 4°C), and frozen at \(-20°C\) until analysis.*

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured, as previously described by Jaffe et al.\textsuperscript{21} in culture medium supplemented with 20% FCS. For experiments, cells were incubated in culture medium containing 1% FCS for 36 hours.

### Quantification of tPA

**tPA activity** present in SVAREC supernatants was quantified by using a chromogenic assay (Spectrolyse/Fibrin, Biopool). Samples were diluted (1:40) and analyzed as described by the manufacturer.

To minimize the differences between animals or HUVEC cell numbers, tPA activity in aortic lysates or in HUVEC supernatants was related to protein content of aortic or HUVEC cell lysates.

Statistical analyses of the data were performed using 1-way ANOVA followed by a protected least significant difference test. Statistical significance was accepted at \(P<0.05\).

### Zymographies

Zymographies were performed on SVAREC supernatants. One microgram of total proteins was mixed with the gel electrophoresis buffer. After separation on 10% SDS-PAGE, the gel was soaked in Triton X-100 (2.5%) for 1 hour and layered onto a fibrin agarose gel containing bovine plasminogen—enriched fibrinogen (7 mg/mL), bovine thrombin (40 U/mL), and agaorgen (1%), as previously described by Loskutoff and Schleef.\textsuperscript{22} Zymograms were allowed to develop for 12 to 48 hours at 37°C. Plasminogen activator inhibitor activity was detected by reverse fibrin zymography with the addition of uPA (final concentration, 0.5 U/mL) to the fibrin agarose underlay.

Photographs of the gel were scanned by an imaging densitometer and quantified using the NIH Image 1.55 software program. Data are expressed as the mean±SEM in arbitrary units.

### Immunoblotting

Immunoblotting of samples obtained from 10-fold concentrated supernatants was carried out by standard techniques. Twenty micrograms of total proteins was mixed with the gel electrophoresis buffer and separated on 10% SDS-PAGE under nonreducing conditions. The proteins were transferred to a nitrocellulose membrane (Bio-Rad). A 10% solution of nonfat dried milk in PBS containing 0.1% Tween 20 (PBST) was used to block nonspecific binding and to dilute the primary and the secondary antibodies. The primary antibody was a polyclonal goat anti-human tPA IgG and a polyclonal goat anti-human PAI-1 (diluted 1:500). After blocking for 1 hour at 37°C, the membrane was incubated for 1 hour at 37°C with the primary antibody, and then the membrane was washed 3 times with PBST, incubated for 1 hour at 37°C with horseradish peroxidase—conjugated secondary antibody (Jackson ImmunoResearch Laboratory), and washed 6 times with PBST. Immunoreactive protein bands were detected by the enhanced chemiluminescence method (ECL kit, Amersham).

Protein concentrations were determined by the method of Bradford using the Coomassie protein assay reagent from Pierce.

**DNA**. The murine tPA cDNA and the human PAI-1 cDNA were labeled with \([\text{32}P]\text{dUTP}\) by in vitro transcription, as described by the manufacturer (Kit Riboprobe Gemini II, Promega), using the T3 polymerase for the tPA cDNA and the T7 polymerase for the PAI-1 cDNA. The membranes were hybridized overnight at 42°C and washed twice with 2× SSC and 0.1% SDS at 25°C and then with

**DNA**.
0.1 × SSC and 0.1% SDS at 60°C. Autoradiography was performed by standard procedures using X-AR5 films (Kodak) and intensifying screens at −80°C. Radioactivity was quantified using an InstantImager (Packard).

Immunofluorescence
Cells, grown on glass coverslips, were fixed and stained after 24-hour incubation with cytochalasin D, nocodazole, lovastatin, GGPP, or C3 exoenzyme. Cells were washed with PBS, fixed in 4% formaldehyde/PBS for 10 minutes, and permeabilized in Triton X-100 (0.1% in PBS) for 5 minutes at room temperature. Cells were incubated either with TRITC-phalloidin (1:1000 in PBS) for 30 minutes at 37°C or with the monoclonal antibody anti-human α-tubulin for 1 hour at 37°C (1:2000 in 1% PBS/BSA) revealed by an FITC-conjugated secondary antibody. After 2 washes in PBS, coverslips were mounted using the Dako Faramount mounting medium. Stained cells were stored in the dark at 4°C until analysis with a Nikon microscope.

Results
In Vivo Effects of Lovastatin on tPA Activity
Rats treated for 2 days with lovastatin (4 mg/kg per day) showed a slight but nonsignificant decrease in the plasma cholesterol level compared with that of vehicle-treated rats (2.42 ± 0.12 versus 2.18 ± 0.2 mmol/L, P = NS). Moreover, a previous study in the laboratory showed that a similar treatment of lovastatin for 15 days induced a significant decrease in plasma cholesterol values. These 2 results suggested that the treatment with lovastatin was effective.

Isolated aortas showed a 3-fold increase in tPA activity compared with aortas of vehicle-treated rats (0.35 ± 0.05 versus 1.05 ± 0.8 IU/mg protein, P < 0.002) (Figure 1). In contrast to tPA, no uPA activity could be detected in aortic lysates.

Characterization of the Effects of Lovastatin on the Fibrinolytic System of Endothelial Cells
To better characterize the effects of lovastatin on the fibrinolytic activity of endothelial cells, we next performed experiments on a rat aortic endothelial cell line (SVARECs) and on human endothelial cells (HUVECs) as untransformed cells. SVARECs, as nontransformed endothelial cells, synthesized and released some PAs to promote clot lysis. In SVAREC supernatants, only tPA was observed. No uPA activity could be detected by zymography or by immunoblot.

The same pattern of activity was observed in HUVEC supernatants. However, tPA activity was higher in SVAREC supernatant than in HUVEC supernatant (2.88 ± 0.22 versus 0.11 ± 0.006 IU).

As described in other systems,17 tPA synthesis by SVARECs and HUVECs was affected by the presence of FCS in the medium. Since the effect of FCS could have masked the effects of the HRI, we performed all subsequent experiments on SVARECs after 12 hours of FCS deprivation, and the cells were incubated in medium without FCS. For HUVECs, which could not be serum-starved, we performed the experiments with 1% FCS, the lowest FCS concentration capable of maintaining the viability of HUVECs for 36 hours. Lovastatin, added to the medium for 48 hours, induced a dramatic increase in tPA activity that was detectable in SVAREC supernatants (control, 2.88 ± 0.22 IU; 25 μmol/L lovastatin, 8.75 ± 0.66 IU; P < 0.001). This effect on tPA secretion was observed in the 1- to 50-μmol/L range, with a maximum at 50 μmol/L (Figure 2A), and was reproduced by
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2 other HRIs, simvastatin and compactin, in the same range of concentrations (Figure 2A).

Studies performed on HUVECs showed that lovastatin similarly increased tPA activity (control, 0.457±0.018 IU/mg protein; 5 μmol/L lovastatin, 1.29±0.117 IU/mg protein; P<0.001). Furthermore, the effects were observed for 10-fold lower concentrations of lovastatin in HUVECs compared with SVARECs (Figure 2B).

Time-course studies showed that the increase of tPA due to lovastatin was already detectable after a short incubation time (3 hours) and increased in magnitude with the incubation time, reaching a maximum after 48 hours of incubation (Figure 3).

The increase in tPA activity induced by lovastatin was attributed to an increase in protein expression and not to an increase of tPA-specific activity, since it was accompanied by an increase of tPA antigen visualized by immunoblot: a single band of 68 kDa was detected by immunoblot analysis in control cells and increased in the presence of lovastatin (Figure 4A). This increase was likely the consequence of increased protein synthesis, inasmuch as tPA mRNA content was induced 2.5-fold after 60 minutes of incubation withLovastatin (25 μmol/L) (Figure 4B).

PAI-1 secretion was also affected by lovastatin, which induced a decrease in PAI-1 activity (Figure 5A) and mRNA (Figure 5B). Because of the poor sensitivity of the method, we could not detect PAI-1 antigen by immunoblot. The decrease in PAI-1 activity occurred for the same concentration range of lovastatin. The concomitant increase in tPA and decrease in PAI-1 activities is likely to be synergistic with respect to the stimulation of the fibrinolytic system by lovastatin.

Reversal of the Effects of Lovastatin by Isoprenoids
The effects of lovastatin on tPA activity and protein expression were completely reversed by mevalonate (500 μmol/L), the synthesis of which is catalyzed by HMG CoA reductase, whereas LDL cholesterol (50 μg/mL) did not (control, 3.49±0.06 IU; lovastatin, 12.76±0.782 IU; lovastatin+mevalonate, 3.53±0.53 IU; and lovastatin+LDL cholesterol, 13.99±0.53 IU; P<0.001). These results suggested that the effects of lovastatin involved a modulation of the mevalonate pathway but that end derivatives of the mevalonate pathway, such as cholesterol, were not involved in tPA modulation.

Among the early derivatives synthesized along the mevalonate pathway, the isoprenoids FPP and GGPP are involved in protein regulation by a posttranslational modification leading to the adjunction of the isoprenoids to the COOH terminal part of the protein. To determine whether the effects of lovastatin were mediated by the isoprenylation of a protein, we incubated cells in the presence of lovastatin and of the 2 isoprenoids FPP and GGPP (Figure 6). FPP and GGPP alone did not modify tPA and PAI-1 activity (data not shown). FPP (15 μmol/L) did not abolish tPA increase, and HFPA (15 μmol/L), a competitive inhibitor of the farnesyltransferase, could not reproduce the effects of lovastatin, suggesting that modulation of a farnesylated protein was not involved in tPA regulation. In contrast with FPP, GGPP (15 μmol/L) completely inhibited the augmentation of tPA and the decrease of PAI-1 activity observed with lovastatin (Figure 6A). tPA protein increase was also prevented by
GGPP, as shown by the immunoblot analysis (Figure 6C), suggesting the involvement of a geranylgeranylated protein in tPA regulation.

Interaction With Intracellular Signaling Pathways
Among the isoprenylated small GTP proteins, the Rho and the Rab proteins are known to be mostly geranylgeranylated.\textsuperscript{25} To determine whether the effects of lovastatin on tPA activity may result from the inhibition of Rho proteins, we incubated cells in the presence of C3 exoenzyme, a specific inhibitor of the Rho proteins.\textsuperscript{26} (Figure 7). C3 exoenzyme (5 μg/mL) induced the same effect as lovastatin on tPA and PAI-1 activity, had no synergistic effects with FPP and lovastatin, and blocked the reversal of the effects of lovastatin by GGPP, suggesting the involvement of Rho proteins. The involvement of other geranylgeranylated proteins different from Rho proteins seemed highly improbable, since C3 exoenzyme completely prevented the effects of GGPP, and GGPP did not modify the effects of C3 exoenzyme on tPA and PAI-1 synthesis.

Modification of the Cytoskeleton
Since Rho proteins are known to regulate the cytoskeletal proteins,\textsuperscript{27} we evaluated whether the modulation of tPA activity could be accounted for by a modification of the cytoskeleton. Cytochalasin D, a well-known disrupter of the actin filaments, dose-dependently (20 nmol/L to 20 μmol/L) induced an increase in tPA activity. This effect was correlated with the progressive disappearance of the cellular actin stress fibers (Figure 8A). In contrast, nocodazole, which disrupts microtubules, had no effect on tPA activity and actin stress fibers (Figure 8B). Lovastatin (25 μmol/L) induced a disruption of the cellular actin stress fibers compared with control cells, without any modification of the microtubule network (Figure 9). The correlation between the organization of actin filaments and the level of tPA was also reproduced with the GGPP, FPP, and C3 exoenzyme (data not shown). GGPP completely prevented the disappearance of actin stress fibers induced by lovastatin, whereas FPP did not. C3 exoenzyme induced the same disruption of the cellular actin filaments as lovastatin and blocked the reversal of the effect by GGPP. These results may suggest that tPA activity was regulated by the cytoarchitecture of the cells and that the induction of tPA synthesis by lovastatin might result from modifications of the actin filaments.
Discussion

In the present study, we demonstrated for the first time a direct action of inhibitors of the HMG CoA reductase on the endothelial fibrinolytic system. Lovastatin and 2 other inhibitors, simvastatin and compactin, induced a dramatic increase in the local fibrinolytic system by stimulating tPA expression and activity, an effect that was potentiated by the inhibition of PAI-1. This effect was dependent on geranylgeranylated proteins, which could be the Rho proteins, and was associated with a disruption of the actin filaments.

Although the concentration of lovastatin used in the present study was higher than that observed in the plasma of patients treated with lovastatin (0.01 to 0.5 μmol/L), the relevance of the effects observed in this study for patients treated with HRI was supported by several facts. First, the results were observed in vivo with doses of lovastatin previously reported in the treatment of hyperlipidemia in different models of hyperlipidemic rats. Second, the concentration of lovastatin needed to induce tPA activity in nontransformed human endothelial cells (HUVECs) was in the same range as that which inhibits the proliferation of smooth muscle cells, glomerular mesangial cells, and fibroblasts. In SVARECs, higher concentrations of HRIs were necessary to observe the same effects. It is likely that these differences could be accounted for by the higher HMG CoA reductase activity present in simian virus 40–transformed cell lines, such as SVARECs, as demonstrated by Larsson.

In contrast to the inhibitory effect of lovastatin on proliferation, which was observed after a long incubation period with the drug (24 and 48 hours), stimulation of fibrinolysis was detectable after treatment as short as 2 days and an incubation time of 3 hours.

The fact that simvastatin and compactin, 2 other HRIs, reproduced the effects of lovastatin indicated that the mechanism of the stimulation was related to the inhibition of the mevalonate pathway (which leads to the synthesis of cholesterol) and of a variety of isoprenoid metabolites: dolichol, ubiquinone, and the isoprenoids FPP and GGPP. The com-

Figure 8. Effect of cytochalasin D and nocodazole on tPA activity and actin filaments. Increasing concentrations of cytochalasin D (0.02 μmol/L to 2 μmol/L) or nocodazole (0.1 μmol/L to 1 μmol/L) were added for 24 hours before supernatants were collected and cells were fixed for immunofluorescence. tPA activity was evaluated by zymography. Organization of actin filaments was analyzed by staining with TRITC-phalloidin. A, Effects of cytochalasin D. B, Effects of nocodazole.

Figure 9. Effect of lovastatin on actin filaments and microtubules. SVARECs were incubated for 24 hours with lovastatin (25 μmol/L) before fixation and analysis by immunofluorescence of TRITC-phalloidin or α-tubulin staining.
The mechanism linking Rho proteins to fibrinolysis is not fully elucidated. An involvement of the cytoskeleton is suspected, since Rho proteins are known to regulate the organization of the cytoskeleton and the formation of actin filaments. Along this line, Fenton et al and Bifulco et al demonstrated that HRIs could modify the cytoskeleton organization and the polymerization of intracellular actin. We confirmed on SVARECs that HRIs induced a disruption of the actin filament network without affecting the microtubes. This effect, which was reversed by GGPP, seemed to depend on activated Rho proteins, since it was blocked by C3 exoenzyme. The effects of HRIs on fibrinolysis, on the one hand, and on the disruption of the actin filaments through the inhibition of Rho proteins, on the other hand, are likely to be linked. Indeed, cytochalasin D, which is known to disrupt actin filaments, induced the same effect as lovastatin and C3 exoenzyme, whereas nocodazole, which disrupts the microtubes, did not. Along the same line, Snyder et al demonstrated a stimulation of tPA by dihydrocytochalasin B in F9 teratocarcinoma cells. Moreover, the extensively studied link between cytoskeleton and urokinase suggests that modulation of the cytoskeleton could be a potent regulator of tPA synthesis. However, although a link between cytoskeleton reorganization and upregulation of tPA by HRI is suspected, further studies are necessary to assess it definitely.

In conclusion, in the present study, we demonstrated a link between an enhancement of the endothelial fibrinolytic system and the inhibition of the mevalonate pathway by the well-known hypocholesterolemic agents lovastatin, simvastatin, and compactin. The action of the HRIs involved an inhibition of geranylgeranylated proteins, probably the Rho proteins, and was associated with a disruption of the cytoskeleton. These effects, which were observed also in vivo, suggest that HRIs, through the enhancement of local plasmin generation, might prevent fibrin or extracellular matrix deposition in arterial plaques and acute thrombosis in the injured vessel, which are 2 major determinants of the progression of atherosclerosis and cardiovascular mortality.

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