HMG CoA Reductase Inhibitors Reduce Plasminogen Activator Inhibitor-1 Expression by Human Vascular Smooth Muscle and Endothelial Cells

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Abstract—The clinical benefit of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) may derive from a qualitative, functional change in atherosclerotic lesions in addition to their lipid-lowering properties. We examined whether statins altered expression of the major determinants of fibrinolytic balance, plasminogen activator inhibitor-1 (PAI-1), and tissue-type plasminogen activator (tPA) in human vascular smooth muscle (SMC) and endothelial (EC) cells. Simvastatin reduced levels of PAI-1 antigen released from SMCs and ECs stimulated with platelet-derived growth factor or transforming growth factor-β (IC₅₀ ≈ 1 μmol/L). Levels of EC-derived tPA increased 2-fold over the same concentrations of simvastatin that inhibited release of PAI-1. Simvastatin’s inhibitory effect was mimicked by C3 exoenzyme and prevented by geranylgeranyl pyrophosphate, but not by farnesyl pyrophosphate, suggesting the involvement of geranylgeranyl-modified intermediates. Decreased PAI-1 antigen was correlated with reduced mRNA transcription and activity of the PAI-1 promoter. By inhibiting expression of PAI-1 from SMCs and ECs while increasing expression of tPA from ECs, simvastatin may alter the local fibrinolytic balance within the vessel wall toward increased fibrinolytic capacity that, in turn, would reduce thrombotic risk after plaque rupture. (Arterioscler Thromb Vasc Biol. 2000;20:556-562.)

Key Words: atherosclerosis ■ HMG CoA reductase inhibitors ■ fibrinolysis ■ plasminogen activator inhibitor-1 ■ tissue plasminogen activator

Lipid-lowering treatment with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or statins, reduces the incidence of acute cardiovascular events and lowers total mortality in patients with either high or “average” levels of cholesterol.1-3 Interestingly, quantitative angiographic studies of cholesterol reduction have revealed only a relatively modest (1% to 2%) change in coronary arterial stenosis in the face of substantial clinical benefit.4 Therefore, statins may improve qualitative, functional features of atheroma, which account for clinical benefits out of proportion to changes in lesion size.

Cellular mechanisms, beyond reduced cholesterol synthesis, may underlie statin’s vasculoprotective effect observed clinically. Statins reduce cellular pools of farnesyl and geranylgeranyl pyrophosphates, metabolites of mevalonate used in posttranslational prenylation of proteins.5-7 Reduced prenylation of GTP-binding proteins, such as Ras and Rho, may contribute to statin’s well-documented effect of inhibiting cell growth.8,9 Recent evidence shows that statins can increase expression of profibrinolytic factors while reducing expression of prothrombotic factors, effects also dependent on the inhibition of prenylated intermediates. Endothelial expression of tissue plasminogen activator (tPA) and nitric oxide is enhanced by treatment with statins, effects that would favor local cellular fibrinolysis.10-12 Conversely, treatment with statins inhibits macrophage expression of the prothrombotic molecule tissue factor.13 Statins may thus alter the local cellular fibrinolytic balance toward increased fibrinolytic potential and, if functional in vivo, promote anti-thrombotic mechanisms by cells within atheroma.

Plasminogen activator inhibitor-1 (PAI-1) is the major physiological inhibitor of the plasminogen activators tPA and urokinase in the regulation of fibrinolytic balance.14,15 Disorders associated with increased thrombosis, such as coronary heart disease, deep-vein thrombosis, and obesity, are associated with increased PAI-1 antigen and activity and, in the case of coronary heart disease, decreased tPA activity.16-18 Indeed, both increased plasma PAI-1 levels and a 4G/5G promoter polymorphism that leads to increased plasma PAI-1 are correlated with risk for recurrent myocardial infarction, usually a thrombotic event.19 Human atherosclerotic lesions also exhibit increases of PAI-1 protein and mRNA, localized to smooth muscle cells (SMCs) and inflammatory cells at the sides and bases of lesions, as well as to the overlying endothelium.20-23 These results suggest that local synthesis of PAI-1 and its balance with plasminogen activators may regulate regional plasmin generation and, in turn, influence
the regional fibrinolytic balance within atheroma. In this study, we investigated whether statins modulate expression of PAI-1 and tPA elaborated by vascular SMCs and endothelial cells (ECs) exposed to inflammatory stimuli. We further investigated potential molecular mechanisms that underlie decreased PAI-1 expression.

Methods

Simvastatin was provided by Merck & Co (West Point, Pa) and activated by alkaline hydrolysis of the lactone moiety, according to the manufacturer's protocol. Human recombinant platelet-derived growth factor (PDGF)-BB was from Genzyme; human platelet-derived transforming growth factor (TGF)-β1 was from Collaborative Biomedical Products. Farnesyl pyrophosphate (Fpp), gera-

nylgeranyl pyrophosphate (GGpp), and C3 exoenzyme were purchased from Biomol.

Cell Culture

SMCs obtained from explanted sections of human saphenous vein were grown in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc.) supplemented with 20 mmol/L HEPEs, 10% fetal calf serum (Hyclone), and 5 mmol/L L-glutamine in a humidified atmosphere of 5% CO₂/95% air. ECs were derived from human saphenous veins as described previously. For experiments, SMCs were placed in serum-free DMEM/Ham’s F12 for 24 hours to reduce exposure to serum components. Cells were then pretreated for an additional 16 hours with various statins in 1.0 mL serum-free medium before challenge with various cytokines. ECs were cultured in serum-free medium 199 containing 0.2% human serum albumin with or without statins for 16 hours and then stimulated with cytokines in 1.0 mL of fresh, serum-free medium.

Western Immunoblot and ELISA Analysis

After the experiments, conditioned media were collected and divided in 2 aliquots: 1 was mixed 4:1 with 5% calf serum (Hyclone), and 5 mmol/L L-glutamine in a humidified atmosphere of 5% CO₂/95% air. ECs were derived from human saphenous veins as described previously. For experiments, SMCs were placed in serum-free DMEM/Ham’s F12 for 24 hours to reduce exposure to serum components. Cells were then pretreated for an additional 16 hours with various statins in 1.0 mL serum-free medium before challenge with various cytokines. ECs were cultured in serum-free medium 199 containing 0.2% human serum albumin with or without statins for 16 hours and then stimulated with cytokines in 1.0 mL of fresh, serum-free medium.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated with RNeasy (Tel-Test) from statin-treated human SMCs cultured in 100-mm² dishes after stimulation with agonists for the indicated times. Ten micrograms of total RNA was fractionated on 1.2% agarose/2.2 mol/L formaldehyde gels and blotted to Hybond nylon filters by capillary transfer. Forty-base pair oligonucleotide probes to human PAI-1 or GAPDH (Oncogene Sciences) were end-labeled with [%³²P]ATP and T4 polynucleotide kinase and purified through G-25 Sephadex columns. Blots were hybridized overnight at 42°C with 2 to 10×10⁶ counts per minute of probe in buffer consisting of 0.5 mol/L NaPO₄, pH 7.2; 7% SDS; and 1% BSA. Blots were washed and exposed to autoradiography film for 1 to 3 days in a cassette containing 2 intensifying screens.

Nuclear Isolation and Run-On Analysis

Human SMCs from 4 confluent 175-cm² flasks per experimental group were collected by brief trypsinization and centrifuged. Cell pellets were resuspended in 5.0 mL ice-cold lysis buffer (10 mmol/L Tris-HCl, pH 7.4; 10 mmol/L NaCl; 3 mmol/L MgCl₂; and 0.5% NP-40), gently triturated, and collected by centrifugation (1000g, 5 minutes). Cell pellets were subjected to 3 additional rounds of resuspension and trituration in lysis buffer. An aliquot stained with trypan blue verified lysis of the cells and revealed intact nuclei largely free of cytoplasmic membranes. Nuclear pellets were resus-

pended in 100 μL of storage buffer (20 mmol/L Tris-HCl, pH 8.1; 75 mmol/L NaCl; 0.5 mmol/L EDTA; 1 mmol/L DTT; and 50% glycerol) and stored at −80°C.

In vitro transcription was performed by mixing 100 μL of nuclei with 100 μL of transcription buffer containing 10 mmol/L Tris-HCl, pH 8.0; 5 mmol/L MgCl₂; 300 mmol/L KCl; 50 μmol/L EDTA; 1 mmol/L DTT; 250 U/mL RNasin; and 0.5 mmol/L each of CTP, ATP, GTP, and 200–μCi [%³²P]UTP. Reactions were incubated at 30°C for 30 minutes and terminated by addition of 20 U of RNase-free DNase I. After proteinase K digestion and phenol/ chloroform extraction, newly transcribed mRNA was precipitated with ammonium acetate and 2.5 volumes of ice-cold ethanol. RNA pellets were briefly dried and resuspended in 500 μL of hybridiza-
tion buffer. Radiolabeled RNA (~10⁷ cpm) was hybridized for 48 hours at 42°C to cDNAs (1 μg) from human PAI-1, β-tubulin, and pGEM vector fixed on Hybond nylon filters. After being washed in 1× SSC, filters were exposed to autoradiography film for 1 to 3 days in cassettes with 2 intensifying screens.

Transient Transfection

The human PAI-1 promoter constructs p800, p187, and p100 that contain the luciferase reporter have been described and were gener-
ously provided by Dr David Loskutoff. Bovine aortic SMCs cultured in 12-well dishes were transfected with 0.25 μg of each reporter construct by the lipofectamine-plus method for 5 hours in Optimized minimal essential medium (Gibco-BRL). Efficiency of transfection (12% to 18%) was not altered by experimental treatments, as assessed by expression of green fluorescent protein directed by the cytomegalovirus promoter. This approach was necessary because of increased β-galactosidase activity (~40%) in bovine aortic SMCs exposed to PDGF without a change in cell number, thus invalidating the use of this reporter for assessing changes in transfection efficiency. After transfections, cells were incubated with simvastatin for 16 hours in serum-free DMEM/F12 medium and then stimulated with PDGF-BB (30 ng/mL) for an additional 18 hours. Cell lysates were collected and luciferase activity measured in an aliquot by using a Lumat LB 9501 luminometer. In any individual experiment, values of luciferase activity from triplicate wells varied <10%.

Statistical Analysis

Statistical differences among groups were tested by Students’ t test (2 groups) or by 1-way ANOVA (≥2 groups). Values are presented as the mean±SEM. A probability value ≤0.05 was considered significant.
Results

Statins Reduce PAI-1 Elaborated by Vascular SMCs and ECs and Increase tPA Released by ECs

Initial experiments investigated whether statins could alter the levels of PAI-1 released by human vascular SMCs or ECs. Two inducers of PAI-1 expression, PDGF and TGF-β, increased levels of PAI-1 released from SMCs by 2.6 ± 0.3- and 2.7 ± 0.3-fold, respectively. Simvastatin, in a concentration-dependent manner, significantly reduced the effect of PDGF and TGF-β on PAI-1 levels (Figure 1A). Immunoblot analysis of conditioned medium confirmed the presence of a 47-kDa band that was increased by stimulation with PDGF and TGFβ and was reduced by increasing concentrations of simvastatin (Figure 1A).

In ECs, TGF-β increased levels of PAI-1 by 2.0 ± 0.1-fold. This increase was also reduced by simvastatin in a concentration-dependent manner (Figure 1B). The plasminogen activator tPA was detected in medium conditioned by ECs but not by SMCs and was not altered by TGF-β stimulation. Of interest, simvastatin increased EC-derived tPA to a maximum of 2-fold over controls at concentrations that inhibited induction of PAI-1 (Figure 1B). Under the serum-free conditions of the experiments, tPA antigen in SMC-conditioned medium was below the detection limit of the ELISA employed (1 to 2 ng/mL). Immunoblot analysis of PAI-1 and tPA confirmed the results obtained by ELISA.

To verify that simvastatin reduced synthesis of PAI-1 protein, SMCs and ECs were metabolically labeled, and PAI-1 was immunoprecipitated from the medium. Simvastatin reduced de novo synthesis of PAI-1 protein by SMCs stimulated by PDGF and TGF-β and by ECs stimulated by TGF-β, without inhibiting total protein synthesis (Figure 2). An additional protein of ~70 kDa from SMCs (visible after longer exposure times) and a protein of ~50 kDa from ECs coprecipitated with PAI-1 and remained constant across experimental groups, verifying that changes in PAI-1 were not due to different levels of loading. It is not known whether these coprecipitating proteins specifically interact with PAI-1 or represent low-affinity interactions with the PAI-1/antibody immune complex. Thus, statins reduced the levels of PAI-1 synthesized from cytokine-stimulated human SMCs and ECs while increasing the levels of tPA released from ECs.

Geranylgeranyl but Not Farnesyl Isoprenoids Reverse Simvastatin’s Inhibitory Effect

Experiments with the substrate of 3-hydroxy-3-methylglutaryl coenzyme A reductase, mevalonate, and the isoprenoid intermediates Gpp and Fpp evaluated their involvement in simvastatin’s inhibitory effects. Mevalonate and Gpp, but not Fpp, prevented simvastatin-induced inhibition of PAI-1 (Figure 3A). Under the same conditions, the uptake of tritiated Gpp and Fpp by the cultured SMCs was similar, thus excluding a difference in cellular entry of the
isoprenes as an explanation for Fpp’s lack of effect (data not shown). The Rho family of small GTP-binding proteins are posttranslationally modified by geranylgeranylation and have been implicated in statin’s mechanism of inhibiting the expression of tissue factor and increasing the expression of type I nitric oxide synthase and tPA.10,12,13 Human SMCs were therefore treated with C3 exoenzyme (15 μg/mL), a transferase that selectively ADP-ribosylates and thus inactivates Rho proteins. We compared the effect of this treatment to that of simvastatin on PAI-1 expression. C3 exoenzyme inhibited PDGF-induced increases of PAI-1, thus reproducing the inhibitory effect of simvastatin (Figure 3B). The addition of GGpp did not reverse the inhibitory effect of C3 exoenzyme, consistent with its mechanism of Rho inactivation.

Statins Reduce PAI-1 Gene Transcription

To explore further the mechanism of statin’s inhibitory effect, Northern blot analysis and nuclear run-on assays were performed to determine whether statins reduced PAI-1 transcription. PDGF stimulation of human SMCs increased two PAI-1 transcripts (3.2 and 2.5 kb) in a time-dependent manner (Figure 4A). Preincubation with simvastatin prevented accumulation of both PAI-1 transcripts. PDGF-induced increases of PAI-1 mRNA were in part due to enhanced gene transcription, which increased 3.2±0.8-fold, on average (n=3), after correction for levels of β-tubulin (Figure 4B). Simvastatin alone had a modest effect and significantly (P≤0.05) inhibited PDGF-induced increases of PAI-1 transcription. Thus, statins reduce PAI-1 protein expression in part by reducing transcription of the PAI-1 gene.

Statins Reduce Activity of the PAI-1 Promoter

Reduced PAI-1 gene transcription suggests that statins may inhibit activity of the PAI-1 promoter. Transient transfection experiments were performed with an 800-bp region of the PAI-1 promoter, previously shown to be sufficient for inducibility by phorbol ester and TGF-β.26,27 Bovine aortic SMCs were used for these experiments, as they have a greater efficiency and reproducibility of transfection compared with human SMCs. Luciferase activity from construct p800, which encompasses 2800 to 171 bp of the human PAI-1 promoter, increased 3.3-fold in response to PDGF. Deleting promoter segments to positions −187 (p187) or −100 (p100) did not reduce the fold induction in response to PDGF (3.3- and 4.2-fold, respectively), but absolute luciferase activity decreased with shorter promoter fragments (Figure 5). Simvastatin significantly reduced PDGF-induced increases of luciferase activity from p800, p187, and p100. Of note, the inhibitory effect of simvastatin on PDGF-induced activity of p100, though significant, was less pronounced than on the p800 and p187 constructs. Transfection with promoterless vector (p19-Luc) showed background levels of luciferase activity across the experimental groups. Statin-induced inhibition of PAI-1 gene transcription may thus stem from reduced activity of the PAI-1 promoter.

Discussion

Clinical trials of statin therapy have demonstrated a reduced incidence of cardiovascular events in the face of relatively modest angiographic changes, suggesting that effects of statins other than on the degree of arterial stenosis may underlie part of their clinical benefit. Increased levels of
PAI-1 both systemically and locally within human atheroma may contribute to the hypercoagulable state noted in hypercholesterolemic individuals and may predispose such subjects to an increased risk of thrombotic complications. This study investigated whether statins alter vascular cell expression of PAI-1 and tPA, the major regulatory proteins of fibrinolytic balance, that may in turn promote increased fibrinolysis.

Statins inhibited expression of PAI-1 from human SMCs and ECs exposed to PDGF or TGF-β and increased expression of tPA from ECs. The results with human ECs support those of Essig et al. who recently reported increased tPA and reduced PAI-1 expression by a transformed rat EC line after treatment with statins. We show here that statins inhibit expression of PAI-1 by human SMCs through a mechanism involving reduced activity of the PAI-1 promoter and transcription of the PAI-1 gene. The concentrations of statins that inhibited expression of PAI-1 (0.5 to 2 μmol/L) correspond closely with those reported to inhibit other cellular functions, such as proliferation and expression of tissue factor, but are well below those reported to induce apoptosis of vascular SMCs (≥10 μmol/L). Concentrations of simvastatin within vascular tissue in vivo have not been measured; however, peak plasma concentrations after administration of 40 mg simvastatin (0.1 to 0.2 μmol/L) approach those required to

Figure 4. A. Simvastatin (simva) reduces PAI-1 mRNA in human SMCs. Cells pre-treated for 16 hours with 2 μmol/L simvastatin or vehicle were challenged with PDGF (30 ng/mL). Total cellular RNA was isolated at the indicated times, and PAI-1 or GAPDH mRNA was detected by Northern blot analysis as described in Methods. Results are representative of 3 separate experiments. B. Simvastatin reduces PAI-1 mRNA transcription. Simvastatin-treated (2 μmol/L) human SMCs were stimulated for 8 hours with PDGF (30 ng/mL). Nuclei were isolated, and a run-on assay was performed as described in Methods. Quantification of PAI-1 transcripts, normalized to levels of β-tubulin, pooled from 3 independent experiments are shown in the lower panel. *Statistical significance of P≤0.05.
inhibit cellular functions in vitro. The current data suggest that statins indeed affect expression of proteins central to the regulation of fibrinolytic balance, independent of lipid lowering in part via reduced transcription.

The reversal of simvastatin’s inhibitory effect by mevalonate and GGpp, but not by Fpp, indicates the requirement of posttranslationally modified in this manner and regulate expression. The Rho family of GTP-binding proteins are geranylgeranylated intermediates in the pathway of PAI-1 expression by ECs and inhibition of tissue factor by macrophages. Because several Rho proteins are inactivated by C3 exoenzyme, additional studies are needed to elucidate which family members contribute to induction of PAI-1 expression.

Simvastatin reduced PDGF-induced transcriptional activity of the PAI-1 gene, as assessed by nuclear run-on analysis. This inhibition likely results from corresponding reductions in the activity of the PAI-1 promoter after treatment with simvastatin. Although absolute promoter activity declined with shorter promoter fragments, PDGF inducibility remained constant down to 100 bp upstream of the transcriptional start site. Simvastatin inhibited PDGF induction of all promoter constructs tested; however, the inhibitory effect on p100, the smallest promoter segment tested, was less pronounced than on the 2 larger promoter constructs, p187 and p800. How simvastatin inhibits PAI-1 promoter activity is not resolved, but the data suggest that simvastatin reduces the activity or binding of transcription factors to sequences in the proximal 187 bp of the PAI-1 promoter. This region encompasses a cAMP response element/activator protein-1 element (or p-box) that resembles the consensus site for both activator protein-1 and cAMP response element binding proteins (TGAGTTCA) and is necessary for promoter activity induced by phorbol ester and serum. Further deletions of the PAI-1 promoter showed that the p-box element is also required for PDGF inducibility (data not shown). However, simvastatin reduced PAI-1 expression induced by several agonists, including interleukin-1, angiotensin II, and TGF-β, the latter utilizing promoter elements distinct from the p-box. This suggests a common mechanism whereby simvastatin decreases PAI-1 promoter activity independent of the inducing stimulus and associated transcription factors.

Despite the incompletely resolved mechanism by which statins reduce expression of PAI-1 from SMCs and ECs while increasing tPA from ECs, the consequences of this finding have particular medical interest. Reducing local expression of PAI-1 by SMCs within vascular lesions while increasing tPA expression by luminal ECs may tip the local fibrinolytic balance toward increased fibrinolytic capacity, which could limit the extent of thrombus formation and fibrin(ogen) deposition that follows plaque rupture. On the other hand, increased local fibrinolysis may also promote extracellular matrix degradation that in turn may destabilize advanced atherosclerotic plaques. Treatment of humans with statins has inconsistent effects on circulating PAI-1 antigen; however, such clinical studies cannot assess local fibrinolytic balance within the vessel wall. Altering the local fibrinolytic balance of SMCs or ECs toward increased fibrinolysis may reduce thrombotic risk, thus contributing to the clinical benefit conferred by treatment with statins.

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References


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