Simvastatin Depresses Blood Clotting by Inhibiting Activation of Prothrombin, Factor V, and Factor XIII and by Enhancing Factor Va Inactivation

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Background—The mechanism of the antithrombotic action of statins is unclear. The aim of this study was to evaluate the effects of simvastatin on the coagulation process at sites of microvascular injury.

Methods and Results—Tissue factor–initiated coagulation was assessed in blood samples collected every 30 seconds from bleeding-time wounds of 17 patients who had advanced coronary artery disease and total cholesterol levels of 224.6±11.8 mg/dL (mean±SEM). Quantitative Western blotting for time courses of fibrinogen depletion and activation of prothrombin, factor V, and factor XIII was performed before and after 3 months of simvastatin treatment (20 mg/d). Simvastatin induced reductions in total cholesterol (23%) and LDL-cholesterol (36%), which were accompanied by significant decreases in the rates of prothrombin activation (16.2±2.1%; \( P = 0.004 \)), formation of \( \alpha \)-thrombin B-chain (27.4±1.8%; \( P = 0.001 \)), generation of factor Va heavy chain (29.7±3.1%; \( P = 0.007 \)) and factor Va light chain (18.9±1.2%; \( P = 0.02 \)), factor XIII activation (19.8±1.3%; \( P = 0.001 \)), and fibrinogen conversion to fibrin (72.2±3%; \( P = 0.002 \)). Posttreatment fibrinopeptides A and B concentrations, determined by using high-performance liquid chromatography, were reduced within the last 30 seconds of bleeding. The 30-kDa fragment of the factor Va heavy chain (residues 307 to 506), produced by activated protein C, and the 97-kDa fragment of the factor Va heavy chain (residues 1 to 643) were released more rapidly after simvastatin treatment. The antithrombotic actions of simvastatin showed no relationship to its cholesterol-lowering action.

Conclusions—Simvastatin treatment depresses blood clotting, which leads to reduced rates of prothrombin activation, factor Va generation, fibrinogen cleavage, factor XIII activation, and an increased rate of factor Va inactivation. These effects are not related to cholesterol reduction. (Circulation. 2001;103:2248-2253.)

Key Words: simvastatin \( \bullet \) thrombin \( \bullet \) factor V \( \bullet \) factor XIII \( \bullet \) fibrinogen

The effectiveness of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) in the prevention of coronary artery disease (CAD) is ascribed not only to reduced cholesterol levels,\(^1,2\) but also to a number of additional effects, including the stabilization of atherosclerotic plaque, improved endothelial function, enhanced fibrinolysis, and antithrombotic actions.\(^3,4\) In patients treated with statins, a marked decrease in thrombin generation has been observed,\(^5-7\) although its detailed characteristics and mechanisms have not been determined. Recent data suggest that statins may modulate the blood coagulation cascade, a series of reactions that proceeds through the assembly of 3 complexes of vitamin K–dependent enzymes and cofactors.\(^8\) The extrinsic coagulation pathway plays a central role in vivo. When factor VIIa encounters tissue factor (TF) exposed at the site of injury, they form the extrinsic tenase on cell membranes. This complex activates factor X and factor IX to their active forms. Factor Xa assembles with factor Va into the prothrombinase complex that converts prothrombin to \( \alpha \)-thrombin. This enzyme activates the cofactors factor V and factor VIII, thereby amplifying blood coagulation. In the presence of thrombomodulin, thrombin also activates protein C, which in turn inactivates factor Va and factor VIIa and shuts down further \( \alpha \)-thrombin formation.\(^9\) \( \alpha \)-Thrombin also cleaves the \( \alpha \) and \( \beta \) chains of fibrinogen, releasing fibrinopeptide A (FPA) and, more slowly, fibrinopeptide B (FPB). The generated fibrin monomers polymerize to form a stable clot. The resistance of the clot to plasmin degradation depends on covalent cross-linking of the fibrin monomers. This process is catalyzed by the active transglutaminase form of factor XIII, which is produced by \( \alpha \)-thrombin cleavage.\(^10\)

Impaired TF expression on cultured human macrophages, which is induced by statins, has been demonstrated in vitro and has been attributed to the inhibition of the TF gene...
induction.\textsuperscript{11} TF-initiated thrombin generation on human monocytes was significantly depressed by simvastatin at concentrations of 10 mmol/L to 10 \mu mol/L.\textsuperscript{12} The effect of statins on factor V, factor XIII, and prothrombin activation, along with factor Va inactivation by activated protein C (APC), has not been reported.

The present study was undertaken to evaluate the effects of 3 months of simvastatin treatment on several coagulation reactions at sites of microvascular injury. The advantage of the model used is that coagulation can be assessed under near-physiological conditions in the presence of all the blood components and the vascular endothelium.

**Methods**

**Materials**

Tris-HCl, KOH, and HEPES were purchased from Sigma; Tween-20 was obtained from J.T. Baker; high-performance liquid chromatography–grade H\textsubscript{2}O and CH\textsubscript{3}CN, HC\textsubscript{1}O\textsubscript{4}, and trifluoroacetic acid were from VWR. Murine monoclonal \(\alpha\)-fibrinogen 3A was raised against the A\textsubscript{a} chain of fibrinogen.\textsuperscript{13} Murine monoclonal \(\alpha\)-factor V\textsubscript{a}c No. 17 and \(\alpha\)-factor V\textsubscript{a}c No. 9 recognize factor Va heavy chain (residues 307 to 506) and factor Va light chain, respectively.\textsuperscript{14} Burro polyclonal \(\alpha\)-prethrombin-1 antibody, which recognizes prothrombin, prethrombin 1, prethrombin 2, prothrombin fragment 2, and \(\alpha\)-thrombin B-chain, was prepared at the Division of Hematology Research, Mayo Clinic.\textsuperscript{15} Rabbit polyclonal \(\alpha\)-factor XIII (D4679), which was raised against the subunit A, was a gift from Dr Gerry Lasser (ZymoGenetics, Seattle, Wash). Horseradish peroxidase–labeled goat antirabbit, antimouse, and antihorse IgG antibodies were purchased from Southern Biotech. Molecular standards were purchased from Gibco-BRL. Chemiluminescent substrate was purchased from NEN Life Science Products Inc.

**Patients**

Seventeen men, aged 39–64 years (mean, 51.6 years), who had documented advanced CAD and hypercholesterolemia, were studied. All patients were required to have (1) total serum cholesterol (TC) between 200 and 250 mg/dL and triglycerides <200 mg/dL, and (2) a previous myocardial infarction (n=10) or hospitalization as a result of unstable angina (n=7) within the past 4 to 36 (mean=12) months before entering the study. Exclusion criteria were secondary hypercholesterolemia, unstable angina, uncontrolled hypertension, symptomatic congestive heart failure, serious concomitant diseases, or treatment with agents that interfere with coagulation profiles. None of the subjects had a history of venous thromboembolism. All patients were treated with aspirin (75 mg/d). Plasma fibrinogen and antithrombin III (AT-III) levels were determined nephelometrically (Dade Behring), along with lipids by standard methods, before and after simvastatin therapy. Simvastatin (Zocor, Merck Sharp, and Dohme) was administered over the course of 3 months at a dose of 20 mg/d. All patients gave informed consent, and the protocol was approved by the University’s Ethics Committee.

**Model of Microvascular Injury**

Evaluation of TF-initiated coagulation at sites of hemostatic plug formation was performed in blood obtained from bleeding-time wounds, which were made with a Simplate II device (Organo Teknika) on the lateral aspect of a forearm.\textsuperscript{16,17} All procedures were performed by the same investigator. Blood was collected into heparinized capillaries every 30 seconds until cessation of bleeding and then mixed with anticoagulants, including sodium citrate, aprotinin, chloromethylketone, and heparin (vol/vol, 1:10), which were provided by an FPA assay kit (Diagnostica Stago). Volumes of samples (range, 10 to 50 \mu L) were recorded. No correlations were found between the volume of the samples and concentrations of the parameters measured. Soluble and insoluble materials were separated by centrifugation at 4°C (20 minutes at 2000 \(g\)). Supernatants were stored at −80°C for further analyses.

**Gel Electrophoresis and Western Blotting**

Sodium dodecyl sulfate polyacrylamide gel analysis was performed according to a modified Laemmli procedure.\textsuperscript{13,18} Samples were separated on 5% to 15% linear gradient gels under reducing or nonreducing conditions and were transferred to nitrocellulose membranes (Bio-Rad) by using semi-dry transfer.\textsuperscript{19} Western blotting was performed with the primary antibodies \(\alpha\)-prethrombin 1 (7.5 \mu g/mL), \(\alpha\)-factor V\textsubscript{a}c No.17 (7.5 \mu g/mL), \(\alpha\)-factor V\textsubscript{a}c No.9 (7.5 \mu g/mL), \(\alpha\)-factor XIII (5 \mu g/mL), and \(\alpha\)-fibrinogen 3A (1.5 \mu g/mL). Time courses of factor Va light and heavy chain generation, prothrombin activation, fibrinogen cleavage, and factor XIII activation were analyzed and quantified by densitometry of immunoblots on a Hewlett-Packard Scanjet 4CT.\textsuperscript{11} Concentrations were estimated from serial dilutions of purified standard proteins by horizontal comparison of sample band density. Relative concentrations were determined by normalizing the data with regard to the maximum. Changes in the reaction rates were analyzed using IGOR Pro Version 3.1 software (WaveMetrics Inc). A mean value was calculated from the compilation of 30-s interval densitometric analysis.

**High-Performance Liquid Chromatography**

**Analysis for Fibrinopeptides**

Peptides from supernatants collected from the last 30-s interval were separated by reverse-phase chromatography, as described previously.\textsuperscript{20} FPA and FPB were identified with matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (linear model, PE Applied Biosystems) and were quantitated as described previously.\textsuperscript{20}

**Statistical Analysis**

All results were expressed as means±SEM unless otherwise stated. Differences between parameters before and after simvastatin treatment were analyzed with the Wilcoxon matched pairs test. Correlations were determined with the Spearman’s rank correlation test. Statistical significance was accepted at a level of \(P<0.05\).

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**Figure 1.** A, Immunoblot analyses of \(\alpha\)-thrombin B-chain (Fila-B, bottom band), prothrombin 2 (pre2, middle band), and prothrombin fragment 1.2 (F1.2, upper band) in the fluid phase of reduced blood samples from a typical patient. A wells are blood samples before simvastatin treatment; B wells are post-treatment samples. B, Kinetics of Fila-B generation in bleeding-time blood from 17 patients. Fila-B concentrations before (○) and after (●) simvastatin treatment are expressed as means±SEM.
Results

Three months of treatment with simvastatin (20 mg/d) resulted in a significant decrease in TC (from 224.6±11.8 mg/dL to 172.7±8.1 mg/dL; P<0.00001) and LDL-cholesterol (from 139.2±5.0 mg/dL to 89.3±4.3 mg/dL; P<0.000001), whereas triglycerides and HDL-cholesterol remained unaltered. There were no simvastatin-induced changes in platelet count (242 000±19 000 cells/µL versus 253 000±13 000 cells/µL), fibrinogen (2.72±0.12 g/L versus 2.68±0.15 g/L), or AT-III (0.23±0.02 g/L versus 0.27±0.02 g/L). Bleeding time became prolonged from 382±17 s to 419±21 s (P=0.04) after the simvastatin treatment, although sample volumes remained unaltered.

Prothrombin and Its Activation Products

The prothrombin activation products α-thrombin B-chain (FIIa-B, relative molecular weight (M_r) =30 000), prethrombin 2, residues 274 to 579 in the prothrombin molecule (pre-2, M_r =36 000), and fragment 1.2 (factor 1.2, M_r =37 000) were detected on immunoblots in all patients ~120 s after incisions (Figure 1, left lane 5A). The mobility of these fragments was identical to that in the whole blood model with the same antibody. Simvastatin treatment resulted in a 60-s delay in the appearance of the prothrombin activation products (Figure 1A, lanes 1B to 9B). Densitometry on immunoblots of α-thrombin B-chain before treatment showed that, after a lag phase, it appeared rapidly and reached a maximum of 40 nmol/L before the cessation of bleeding (Figure 1B). Simvastatin decreased the rate of thrombin formation by 27.4±1.8% (0.237 nmol·L^{-1}·s^{-1} versus 0.179 nmol·L^{-1}·s^{-1}; P=0.001). An identical pattern was found in the case of prethrombin 2 and factor 1.2 generations (data not shown). There was no relationship between simvastatin-induced reductions in thrombin B-chain generation and reductions in TC (Figure 2A) or LDL-cholesterol (Figure 2B).

Prothrombin concentration decreased to 10% to 20% of the initial value (~1.36±0.07 µmol/L) by the last 30-s interval. Prothrombin consumption was delayed by 60 s after simvastatin administration. The rate of prothrombin disappearance from blood after simvastatin administration was decreased by 16.2±2.1% (P=0.004; data not shown).
Factor V/Va
Simvastatin treatment led to a delay (30 to 60 s) in the appearance of both factor Va heavy chain (Mr = 105 000) and factor Va light chain (Mr = 74 000), as illustrated in Figure 3A (lanes 1 to 9B versus lanes 1 to 7A). Generation of factor Va heavy chain after treatment was slower by almost 29.7 ± 3.1% (P = 0.007), and that of factor Va light chain was 18.9 ± 1.2% slower (P = 0.02), as depicted in Figure 3B. After simvastatin treatment, factor Va heavy chain and light chain concentrations in the last sample (Figure 3A, lane 9B) were 2.9 ± 0.12 nmol/L and 2.7 ± 0.13 nmol/L, respectively, whereas before simvastatin, a mean value for factor Va heavy chain was ≈ 4.2 ± 0.18 nmol/L and factor Va light chain ≈ 3.8 ± 0.15 nmol/L. Overall, these results suggest that production of the light chain is the limiting step in factor Va generation in bleeding-time blood. Factor V (Mr = 330 000) before treatment was complete at ≈ 20 s (Figure 4, lane 4A). In simvastatin-treated patients, this thrombin-dependent reaction was delayed by 60 s (Figure 4).

Factor Va Inactivation
The characteristic fragments of factor Va heavy chain produced by APC were visualized by using monoclonal antibodies. The terminal APC degradation product of factor Va heavy chain (Mr = 30 000), formed as a result of cleavages at Arg 506 and Arg 306,13 migrated identically with a factor Va inactivation product standard (Figure 3C) produced in bleeding-time blood. This fragment was generated more rapidly (by 90 s) after simvastatin treatment (Figure 3C). On reduced gels, a 97-kDa fragment of factor Va heavy chain was found both before (Figure 3D, lanes 1A to 7A) and after simvastatin treatment (Figure 3D, lanes 1B to 7B). A fragment of identical mobility was observed by Hockin et al21 in experiments in which factor Va was incubated with thrombin in the presence of cultured human umbilical vein endothelial cells. This product is the result of an endothelial cell–dependent thrombin inactivation of factor Va by cleavage at Arg 643. As with the factor Va heavy chain, the level of the 97-kDa fragment increased over time. Simvastatin treatment enhanced this factor Va inactivation mechanism. The rate of factor Va inactivation was unrelated to the simvastatin-induced reduction in TC or LDL-C.

Factor XIII/Factor XIIIa
Before treatment, factor XIII activation was complete at ≈ 20 s (Figure 4, lane 4A). In simvastatin-treated patients, this thrombin-dependent reaction was delayed by 60 s (Figure 4).
creased. Simvastatin treatment did not alter the retention of FPA, desAla FPA (an NH2-terminal truncated form), and full length FPA and P-FPA (phosphorylated at Ser 3), and both FPA and FPB were seen, but des-Arg FPB (COOH-terminal cleavage) was not. Simvastatin treatment did not alter the retention times of the FPA and FPB generated; however, both FPA and FPB levels were significantly depressed. Cumulative experiments showed that the mean FPA concentration was decreased by 25%, from 4.2±0.21 μmol/L to 3.1±0.16 μmol/L (P=0.02), whereas FPB levels fell from 0.8 μmol/L to levels below the detection limit. Two unidentified peaks labeled I and II (Figure 5) were detected. Peak I increased after simvastatin treatment while peak II decreased. The identification of these peaks is currently in process.

Fibrinogen
At baseline, depletion of fibrinogen from solution was complete after ≈150 s. After the administration of the drug, complete fibrinogen consumption occurred 60 s later and at a much slower rate (72.2±3%; P=0.002) when compared with baseline values (data not shown). Cholesterol levels showed no correlation with the rate of fibrinogen consumption.

Fibrinopeptides
Figure 5 represents a high-performance liquid chromatography (HPLC) elution profile of the fibrinopeptides isolated from the fluid phase of blood of one of the patients and then identified by MALDI-TOF MS (data not shown). All 3 forms of FPA, desAla FPA (an NH2-terminal truncated form), P-FPA (phosphorylated at Ser 3), and full length FPA and FPB were seen, but des-Arg FPB (COOH-terminal cleavage) was not. Simvastatin treatment did not alter the retention times of the FPA and FPB generated; however, both FPA and FPB levels were significantly depressed. Cumulative experiments showed that the mean FPA concentration was decreased by 25%, from 4.2±0.21 μmol/L to 3.1±0.16 μmol/L (P=0.02), whereas FPB levels fell from 0.8 μmol/L to levels below the detection limit. Two unidentified peaks labeled I and II (Figure 5) were detected. Peak I increased after simvastatin treatment while peak II decreased. The identification of these peaks is currently in process.

Discussion
Our results indicate that simvastatin treatment leads to multiple antithrombotic effects at sites of microvascular injury. In CAD patients with TC between 200 and 250 mg/dL, 3 months of simvastatin treatment was associated with a significant delay in and attenuation of the activation of prothrombin, factor V, factor XIII, and fibrinogen. The depressed factor V and factor XIII activation and thrombin-mediated fibrinogen cleavage induced by simvastatin are presumably the consequence of decreased prothrombin activation. Our data provide valuable insights into mechanisms that underlay antithrombotic actions of statins.

The flowing blood model used here can be compared with the TF-initiated coagulation model with nonanticoagulated whole blood, in which the intrinsic pathway is blocked by corn trypsin inhibitor. In the latter system,12 blood clots are formed at constant composition, whereas in the flowing blood system, new reactants are provided continuously to the wound site. Because increased flow rates can falsely lower concentrations of any product, total yield in each interval was calculated, revealing similar simvastatin-induced differences compared with those based on concentrations (data not shown). Comparisons are possible because the same primary antibodies used here for Western blots were also used by Rand et al.12 The increased rates of the reactions in the present model most probably are a consequence of increased levels of TF after vascular injury.22 It might be speculated that the attachment of fibrinogen and prothrombin to exposed subendothelial compounds also contributes to a rapid depletion of these coagulation factors from the pretreatment bleeding-time blood.23

We demonstrated significant reductions in FPA and FPB levels in CAD patients with borderline high hypercholesterolemia. This indicates that both thrombin-mediated cleavages in the fibrinogen molecule are impaired by simvastatin. Hence, fibrin formation is also impaired. In a model of nonanticoagulated whole blood, the complete release of FPA and activation of factor XIII occurred within 5 minutes.20 In the present model, these events occurred within 2 minutes. The unidentified species (I and II) detected by HPLC are suspected to be fibrinogen-related cleavage products resulting from the correlation with FPA and FPB.
Prolongation of the lag phase in α-thrombin B-chain generation in bleeding-time blood also indicates that simvastatin may diminish TF expression/activity. The duration of the lag phase is determined by concentrations of the complex TF-factor VIIa and the tissue factor pathway inhibitor. Levels of prothrombin and AT-III, which significantly influence thrombin generation, remained within the normal range (50% to 150%) during simvastatin treatment and, therefore, cannot explain its antithrombotic effects reported here. Because TF expression is also regulated by TF pathway inhibitor, it will become important to test the influence of simvastatin on this anticoagulant.

The present study provides the first evidence for the formation of the 97 000 fragment (residues 1 to 643) of factor Va heavy chain in vivo, which is believed to be the product of thrombin action on factor Va, expressed in the presence of platelets and endothelial cells. Amounts of this product in bleeding-time blood increased after simvastatin administration, although thrombin concentrations were reduced. Therefore, cleavage of factor Va heavy chain at position 643 may not be catalyzed exclusively by thrombin.

In the microvasculature, in which thrombomodulin concentrations are presumed to be high relative to the blood volume, the appearance of factor Va degradation products occurred much faster than during clotting of the whole blood. A novel finding is that simvastatin induces increased rates of factor Va inactivation by APC, which suggests that the drug modulates the anticoagulant protein C pathway through increased expression of thrombomodulin or its activity. It is also possible that simvastatin affects the amount of active thrombomodulin released in loco from platelets.

Our data provide evidence that the antithrombotic actions of statins are not related to their cholesterol-lowering effects. A possible mechanism by which simvastatin affects coagulation is through the inhibition of the synthesis of isoprenoids such as farnesyl and geranylgeranyl pyrophosphates, which are substrates for posttranslational modification and isoprenylation of numerous intracellular proteins. Suppression of isoprenoid production by simvastatin may lead to decreased expression of TF in the endothelium and/or subendothelium, although such an effect of statins was found only in monocytes and macrophages. Fenton et al. put forward a hypothesis that decreased TF expression, combined with the downregulation in cell signaling after thrombin activation of protease activated receptor-1, may explain antithrombotic properties of HMG-CoA reductase inhibitors.

In conclusion, we present evidence for impaired activation of prothrombin, factor V, factor XIII, and enhanced factor Va inactivation by APC and impaired fibrinogen proteolysis after 3 months of simvastatin treatment in CAD patients with borderline-high hypercholesterolemia. Such a concerted influence of simvastatin on the clotting cascade seems to be independent of its lipid-lowering action and may be the result of depressed isoprenoid production. It may partly explain the early clinical benefits offered by simvastatin treatment to CAD patients.

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