Inhibition of 3-Hydroxy-3-Methylglutaryl Coenzyme A (HMG CoA) Reductase Blunts Factor VIIa/Tissue Factor and Prothrombinase Activities via Effects on Membrane Phosphatidylserine

Dennis J. Dietzen, Keith L. Page, Tina A. Tetzloff, Alan Bohrer, John Turk

Objective—3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) exhibit antithrombotic properties that are independent of reductions in circulating LDL cholesterol. We hypothesized that these antithrombotic properties are mediated by membrane alterations secondary to disrupted lipid metabolism.

Methods and Results—EA.hy926 cells were incubated in the presence of 1 μmol/L atorvastatin supplemented with fetal bovine serum or lipid-depleted serum mixtures. Lipid restriction alone had no effect on cell lipid composition but when atorvastatin was included, phosphatidylserine, sphingomyelin, and cholesterol were reduced by 50% while ceramide content decreased by 70%. These changes in lipid composition did not alter the association of decay accelerating factor or tissue factor with lipid rafts. Atorvastatin in combination with lipid restriction reduced factor VIIa/tissue factor activity by as much as 75% but did not alter tissue factor expression. Prothrombinase activity was reduced to an extent similar to factor VIIa/tissue factor. Mevalonic acid but not LDL reversed the observed changes in lipid content and prothrombinase activity induced by atorvastatin. These findings were confirmed in primary cells.

Conclusions—Inhibition of HMG-CoA reductase limits exposure of phosphatidylserine at the cell surface by restricting the cellular pool of mevalonate-derived isoprenoids. This membrane alteration restricts the activity of proteolytic enzyme complexes that propagate the coagulation cascade. (Arterioscler Thromb Vasc Biol. 2007;27:690-696.)

Key Words: Atorvastatin ■ phosphatidylserine ■ tissue factor ■ prothrombinase ■ membrane

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, collectively referred to as “statins,” inhibit de novo cholesterol synthesis and stimulate LDL uptake by the liver. Many beneficial effects of statins have been ascribed to hypolipidemic properties,1–3 but other beneficial effects of the statins are not easily linked to cholesterol reduction. Stabilization of atherosclerotic plaques, inhibition of vascular smooth muscle cell (VSMC) proliferation, and improvements in endothelial nitric oxide production are examples of these “pleiotropic” effects.4–6 Statins also exert a poorly understood anticoagulant effect.7–9

The extrinsic coagulation cascade is propagated by proteolytic complexes that form on the surface of cellular membranes.10 The process is initiated when tissue factor (TF) interacts with circulating factor VIIa (fVIIa) and generates factor IXa (fIXa) and factor Xa (fXa). The tenase complex (fVIIIa and fIXa) accelerates the production of fXa while the prothrombinase complex (factors Va and Xa) generates thrombin. Each of these protease complexes requires calcium and anionic phospholipid such as phosphatidylserine (PS) for full activity.

Previous studies of statin treatment on blood coagulation have shown that statins blunt procoagulant and enhance anticoagulant processes. Statins blunt expression of TF by inflammatory mediators such as lipopolysaccharide (LPS), interleukins, or tumor necrosis factor (TNF-α),7 enhance degradation of factor Va,9 and reduce endothelial expression of plasminogen activator inhibitor (PAI)-1.11 We hypothesize that statins modulate coagulation via effects on the plasma membrane. First, statins may disrupt lipid rafts, cholesterol rich membrane domains important for maintaining membrane bilayer asymmetry.12 Second, statin treatment may indirectly alter PS synthesis.

Statins reduce cellular isoprenoids and induce synthesis of phosphatidylcholine (PC).13,14 PS is synthesized from PC via a choline/serine exchange reaction15 so statins may also impact membrane PS. The contribution of these metabolic perturbations to the control of coagulation has not been investigated.

The goal of the present study was to define the impact of atorvastatin (Lipitor) on the control of phospholipid-dependent protease activities responsible for fibrin formation.
We investigated the effects of atorvastatin on (1) basal expression of TF antigen, (2) the structure of lipid rafts, (3) cellular phospholipid content, and (4) cellular PS exposure. We find that statins do not alter basal TF expression or raft structure but impact both basal and stimulated exposure of PS limiting FVIIa/TF and prothrombinase activities.

Materials and Methods

Reagents and Cells
EA.hy926 cells were obtained from Dr Cora-Jean Edgell (University of North Carolina, Chapel Hill) and cultured in DMEM with fetal bovine serum (FBS). Adult human dermal fibroblasts were obtained from Cambrex (East Rutherford, NJ) and cultured in FGM-2. All cells were cultivated in a 5% CO₂, 95% humidified air atmosphere at 37°C. Lipid-depleted serum (LDS) was prepared by silicic acid treatment.17 Atorvastatin was a gift from Pfizer, Inc. (Groton, Conn). DL-mevalonolactone, methyl-β-cyclodextrin, annexin V, calcium ionophore (A23187), n-octyl-β-D-glucopyranoside (octyl glucoside), and anti-mouse IgG-phycoerythrin conjugate were from Sigma-Aldrich (St. Louis, Mo). Human prothrombin and factor V were obtained from Hematologic Technologies (Essex Junction, Vt). Human factor Xa was a gift from Enzyme Research Laboratories (South Bend, Ind). Amidolytic substrates for factor Xa (Spectrozyme Xa) and thrombin (Spectrozyme TH) were from American Diagnostica (Stamford, Conn). Lipid standards were obtained from Avanti Polar Lipids (Alabaster, Ala). Human LDL was obtained from Lee VitaTech (Stamford, Conn). Human prothrombin and factor V were obtained from Hematologic Technologies (Essex Junction, Vt). Human factor Xa was a gift from Enzyme Research Laboratories (South Bend, Ind). Amidolytic substrates for factor Xa (Spectrozyme Xa) and thrombin (Spectrozyme TH) were from American Diagnostica (Stamford, Conn). Lipid standards were obtained from Avanti Polar Lipids (Alabaster, Ala). Human LDL was obtained from Lee VitaTech (Stamford, Conn). Human prothrombin and factor V were obtained from Hematologic Technologies (Essex Junction, Vt). Human factor Xa was a gift from Enzyme Research Laboratories (South Bend, Ind). Amidolytic substrates for factor Xa (Spectrozyme Xa) and thrombin (Spectrozyme TH) were from American Diagnostica (Stamford, Conn). Lipid standards were obtained from Avanti Polar Lipids (Alabaster, Ala). Human LDL was obtained from Lee VitaTech (Stamford, Conn). Human prothrombin and factor V were obtained from Hematologic Technologies (Essex Junction, Vt). Human factor Xa was a gift from Enzyme Research Laboratories (South Bend, Ind). Amidolytic substrates for factor Xa (Spectrozyme Xa) and thrombin (Spectrozyme TH) were from American Diagnostica (Stamford, Conn). Lipid standards were obtained from Avanti Polar Lipids (Alabaster, Ala). Human LDL was obtained from Lee VitaTech (Stamford, Conn). Human prothrombin and factor V were obtained from Hematologic Technologies (Essex Junction, Vt). Human factor Xa was a gift from Enzyme Research Laboratories (South Bend, Ind). Amidolytic substrates for factor Xa (Spectrozyme Xa) and thrombin (Spectrozyme TH) were from American Diagnostica (Stamford, Conn). Lipid standards were obtained from Avanti Polar Lipids (Alabaster, Ala). Human LDL was obtained from Lee VitaTech (Stamford, Conn). Human prothrombin and factor V were obtained from Hematologic Technologies (Essex Junction, Vt). Human factor Xa was a gift from Enzyme Research Laboratories (South Bend, Ind). Amidolytic substrates for factor Xa (Spectrozyme Xa) and thrombin (Spectrozyme TH) were from American Diagnostica (Stamford, Conn). Lipid standards were obtained from Avanti Polar Lipids (Alabaster, Ala). Human LDL was obtained from Lee VitaTech (Stamford, Conn). Human prothrombin and factor V were obtained from Hematologic Technologies (Essex Junction, Vt). Human factor Xa was a gift from Enzyme Research Laboratories (South Bend, Ind). Amidolytic substrates for factor Xa (Spectrozyme Xa) and thrombin (Spectrozyme TH) were from American Diagnostica (Stamford, Conn). Lipid standards were obtained from Avanti Polar Lipids (Alabaster, Ala). Human LDL was obtained from Lee VitaTech (Stamford, Conn).

Phospholipid, Cholesterol, and Lipid Phosphate Analysis
Cells were suspended in 0.63% LiCl in silanized tubes and washed two times to remove traces of inorganic phosphate (Pᵢ). Total phospholipids were extracted according to Bligh and Dyer18 and determined using a Finnigan TQ-7000 tandem mass spectrometer with electrospray ionization.19–22 Phosphatidylcholine (PC) and sphingomyelin (SM) were quantified using dimyristoyl PC as the internal standard. Ceramide (CER) was identified using C8:0 CER as internal standard. Phosphatidylethanolamine (PE) and phosphatidylserine (PS) were determined using dimyristoyl PE and PS as internal standards, respectively. Lipid phosphate was released by acid hydrolysis and determined using malachite green and ammonium molybdate according to Zhou and Arthur.23 Cholesterol was determined using an enzymatic procedure (Ruchem) on ether extracts in the presence of 10 mg of Triton X-100.

Determination of Factor VIIa/TF and Prothrombinase Activities
Factor VIIa/TF activity was measured on an intact monolayer of cells as in Dietzen et al.12,24 Prothrombinase activity was likewise assessed on an intact cell monolayer using buffer containing 21 mmol/L Hepes (pH 7.4), 137 mmol/L NaCl, 5 mmol/L KCl, 0.75 mmol/L Na₂HPO₄, 5.5 mmol/L glucose, 2 mmol/L CaCl₂, 0.5 µmol/L thrombin, 1 mmol/L fXa, and 0.5 mmol/L thrombin substrate. Reactions were initiated with 5 mmol/L factor V and followed at 405 mmol/L for 60 minutes. Exposure of membrane PS was achieved using methyl-β-cyclodextrin17 or calcium ionophore (A23187).

TF ELISA
Plates were coated with 5 µg/ml monoclonal anti-human TF and blocked in buffer containing 17 mmol/L sodium borate, 0.12 mol/L NaCl, 0.05% Tween-20 1 mmol/L EDTA, 0.25% bovine serum albumin, and 0.05% sodium azide. Lysates prepared in 60 mmol/L octyl glucoside were applied overnight and the plate was washed with distilled water before sequential addition of anti-TF polyclonal antibody and anti-rabbit immunoglobulin conjugated to alkaline phosphatase. Bound alkaline phosphatase activity was detected using p-nitrophenyl phosphate as substrate. Recombinant human TF was used as a standard.

Miscellaneous
Flow cytometry for cell surface TF was performed as described in Dietzen et al.12 Cell numbers were determined directly by flow cytometry or by incubation in culture wells with WST-1 (Oncogene Research). Protein was determined using BCA (Pierce). Plasma membrane was isolated on a Percoll gradient25 and lipid rafts were isolated on the basis of detergent insolubility and buoyant density according to our previous reports.12 The statistical significance of differences between treatment conditions was assessed by the use of an unpaired t test (Microsoft Excel, 2003). Relevant one-tailed probability values are cited in Figure and Table legends.

Results

Atorvastatin and Lipid Restriction Do Not Alter Cell Growth
EA.hy926 cells are the fusion product of HUVECs and the A549 lung carcinoma cells that constitutively express TF. The cellular effects of statins are difficult to define because they are potentially toxic and they induce expression of HMG-CoA reductase and the LDL receptor.26–28 We therefore used pharmacologically relevant concentrations of atorvastatin (1 µmol/L) and limited access to serum lipid to achieve a significant impact on cellular cholesterol. Normal FBS used during the course of this study contained 0.54±0.13 mmol/L (21±5 mg/dL) cholesterol (mean±SD, n=6). Cholesterol was not detectable in LDS. A 50% and 75% reduction in exogenous sterol was achieved using mixtures of serum consisting of 1:1 and 1:3 mixtures of FBS to LDS (v/v), respectively. The serum mixtures always constituted 10% of the media by volume. Over 72 hours, cells treated with 1 µmol/L atorvastatin and reduced serum lipid grew at identical rates to those incubated with FBS but without atorvastatin (Figure 1). Although the net size of the cell population was not impacted, these data might be explained by an increase in both cell growth and death. To exclude this possibility we assessed LDH activity in conditioned media and found no differences between the various culture conditions, indicating similar rates of cell turnover.

Atorvastatin and Lipid Restriction Do Not Disrupt Lipid Rafts
Cholesterol extraction from cells using cyclodextrin destabilizes lipid rafts. It is widely assumed, but not known, if...
restriction of de novo cholesterol synthesis with statins is similarly capable of disrupting rafts. We therefore assessed the impact of atorvastatin on cellular sterol levels and the integrity lipid rafts. We assessed the association of GPI-anchored proteins (eg, DAF, CD59) with rafts as a marker of integrity. As shown in Figure 2 (middle), lipid restriction alone did not reduce cell cholesterol. Atorvastatin reduced cell cholesterol in a fashion dependent on the extent of serum lipid depletion. The maximum decrease in cellular cholesterol achieved did not measurably alter raft integrity.

**Statin Treatment and Sterol Restriction Reduce fVIIa/TF Activity but Not TF Expression**

Statins blunt induction of tissue factor expression induced by inflammatory mediators,7,8,29,30 but the impact of statins on basal TF expression is less clear. We examined the effect of atorvastatin and sterol restriction on the level of TF antigen and its cofactor activity with fVIIa in EA.hy926 cells (Figure 3). Analysis by Western blot, ELISA, and flow cytometry indicated that statin treatment alone or in combination with lipid restriction did not significantly alter cellular levels of TF antigen or its exposure at the cell surface. Statin treatment and lipid restriction, however, did reduce fVIIa/TF activity in untreated cells and cells treated with cyclodextrin to decrypt TF cofactor activity.12 Activity measurements were performed in the presence of saturating amounts of an inhibitory anti-TFPI antibody to obviate the influence of TFPI. Cells exposed to atorvastatin or lipid restriction alone supported similar basal rates of fVIIa/TF activity which doubled following treatment with cyclodextrin. Atorvastatin-treated cells with limited access to serum lipid exhibited suppressed fVIIa/TF activity that was dependent on the degree of lipid restriction. Cells exposed to 1:1 FBS/LDS exhibited basal and decrypted fVIIa/TF activities that were reduced by 30% to 40% with virtually no evidence of encrypted activity.

**Statin Treatment and Lipid Restriction Reduce Phosphatidylserine Exposure**

Results presented in Figure 3 cannot be explained by altered expression of TF or TFPI. We therefore hypothesized that fVIIa/TF activity was modulated by the membrane environment. To assess the impact of atorvastatin and lipid restriction on PS exposure, we established conditions in which prothrombinase activity was proportional to the amount of exposed PS (see supplemental Figure I, available online at
Under these conditions, prothrombinase activity was directly proportional to cell number and the amount of exposed PS (titrated using annexin V). Cells treated with atorvastatin and lipid restriction displayed decreased prothrombinase activity that was similar to the decrease in fVIIa/TF activity (Figure 4, upper panel). Prothrombinase activity was decreased by 30% and 40%, respectively, when exogenous access to lipid was reduced by 50% and 75%. There was no difference in cell number between wells (assessed by parallel incubation with WST) and no change in the sensitivity of cells to saturating amounts of annexin V. These data indicate that altered exposure of PS accounts for decreased TF cofactor activity.

**Atorvastatin Induces Changes to Cellular Phospholipid Pools**

Changes to fVIIa/TF appear to be directly related to membrane alterations, so we assessed the impact of atorvastatin and lipid restriction on cellular phospholipid profiles. Phospholipid concentrations (normalized to lipid phosphate) of control cells were set to one and relative changes to phospholipid activity was decreased by 30% and 40%, respectively, when exogenous access to lipid was reduced by 50% and 75%. There was no difference in cell number between wells (assessed by parallel incubation with WST) and no change in the sensitivity of cells to saturating amounts of annexin V. These data indicate that altered exposure of PS accounts for decreased TF cofactor activity.

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Mechanism of Membrane Alterations Induced by Atorvastatin

Atorvastatin induced drastic changes to the cellular pools of PS, SM, and CER. To determine whether changes to the PS pool were secondary to decreased SM, we exposed cells to PS, SM, and CER. To determine whether changes to the PS membrane were secondary to decreased SM, we exposed cells to 25 μmol/L myriocin, an inhibitor of sphinganine:palmitoyl-transferase, the first committed step of SM synthesis. Myriocin decreased cellular SM by 70% and cellular CER concentrations to undetectable levels but did not impact cellular PS content (Table 1). These changes in lipid content did not alter cell growth (not shown) and did not alter prothrombinase activity (Figure 4A). Thus, changes to PS content and exposure are not mediated by changes to the SM pool.

We also used mevalonic acid and purified human LDL to define the mechanism behind the effect of atorvastatin on membrane PS exposure. Media was supplemented with 200 μmol/L mevalonic acid or LDL cholesterol at 0.052 mmol/L (2 mg/dL) or 0.52 mmol/L (20 mg/dL). These LDL concentrations were designed to mimic the sterol concentrations in media containing 10% or 100% FBS, respectively. Mevalonic acid reversed changes to cellular phospholipid content and prothrombinase activity (Table 1 and Figure 4, upper panel) whereas LDL restored cellular cholesterol levels but not prothrombinase activity in unstimulated cells or those treated with A23187 to induce PS exposure (Figure 4, lower panel). Taken together, these data indicate that the effects of atorvastatin observed in our study are mediated by a mevalonate-derived intermediate(s) and not by cholesterol itself.

Finally, we extended our observations in EA.hy926 cells to primary cells. Normal adult human fibroblasts were exposed to the same atorvastatin concentrations but without lipid restriction because these cells were already cultured in only 2% FBS as opposed to 10%. Basal prothrombinase activity and that decrypted with A23187 were suppressed in a similar fashion to that observed with the immortalized EA.hy926 cultures (Figure 4, lower panel).

**Discussion**

Molecular explanations for statin pleiotropy are generally lacking. Among the more puzzling effects of statins is their capacity to depress blood clotting. We recently showed that cholesterol-rich membrane domains known as rafts are necessary to maintain the transbilayer gradient of PS. Prompted by the obvious impact of statins on cholesterol metabolism and reports linking statins to altered phospholipid metabolism, we investigated the potential for atorvastatin to mediate

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**TABLE 1. Effect of Lipid Restriction, Atorvastatin, Mevalonate, and Myriocin on Cell Phospholipid Content**

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<thead>
<tr>
<th>FBS:LDS</th>
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<tr>
<td>PC</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>0.7 ± 0.3</td>
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<td>PE</td>
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<td>PS</td>
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<td>SM</td>
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<td>CER</td>
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</table>

Where indicated, EA.hy926 cells were incubated for 72 hours in the presence of 1 μmol/L atorvastatin, 200 μmol/L mevalonate, or 25 μmol/L myriocin in the presence of 10% FBS or the indicated ratio of FBS to lipid-depleted FBS. Data presented are ratios to the lipid content of control cells normalized to lipid phosphate content from three lipid extracts (±SD). *P<0.05 vs control; **P<0.001 vs control; NS indicates not significantly different from control cells; ND, none detected.

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**TABLE 2. Effect of Lipid Restriction and Atorvastatin on Plasma Membrane Composition**

<table>
<thead>
<tr>
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<td>PE</td>
<td>25.5±9.7</td>
<td>18.4±5.8</td>
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<td>9.4±0.8</td>
<td>9.7±2.8</td>
<td>7.4±1.6*</td>
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<tr>
<td>SM</td>
<td>34.0±5.8</td>
<td>36.8±7.0</td>
<td>29.3±1.1</td>
<td>40.1±15.7</td>
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<tr>
<td>CER</td>
<td>5.8±3.0</td>
<td>14.4±3.7</td>
<td>8.8±4.8</td>
<td>8.8±2.5</td>
</tr>
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</table>

Data are % by weight of each lipid species from 3 plasma membrane preparations (±SD). * P<0.05 vs control plasma membrane.
thrombosis via effects on membrane lipids. The major findings of this study are twofold. First, atorvastatin treatment in combination with lipid restriction alters cellular membranes but does not disrupt lipid rafts nor alter basal expression of TF. Second, a reduction in plasma membrane PS is associated with decreased fVIIa/TF and prothrombinase activities through a mechanism mediated by mevalonate-derived intermediates, not cholesterol.

We were unable to metabolically disrupt lipid rafts in the present study using concentrations of statins that might be expected in vivo and achieving a 50% reduction in cellular cholesterol. Only acute treatment of cells with supraphysiologic concentrations (up to 100 μmol/L) of statins has been shown to alter the properties of rafts. Furthermore, repletion of cellular cholesterol using LDL did not reverse the decrease in prothrombinase activity. It is highly unlikely, therefore, that statins structurally alter rafts (in vivo) and that rafts play a role in the membrane alteration we define in the present study.

Although lipid rafts were not perturbed by atorvastatin treatment, we observed significant changes to the cellular phospholipid profile. The cellular content of SM, CER, and PS were all significantly reduced while PC and PE content did not change appreciably. Alterations of SM and CER were limited to the intracellular membrane pool, but the reduction in PS was reflected in the plasma membrane as well (Table 2). Reductions in cellular PS may be the result of decreased synthesis (via serine/choline exchange) or enhanced degradation (via PS decarboxylase). A thorough characterization of the expression and kinetic properties of the enzymes involved in PS synthesis and degradation will be required to address the mechanisms responsible for altered membrane PS.

The changes to SM, CER, and PS induced by atorvastatin appear unrelated. A specific inhibitor of SM synthesis, myriocin, achieved reductions of SM and CER consistent with those we observed using atorvastatin and lipid restriction. However, the reductions in SM and CER were not accompanied by changes in PS content and did not alter the cell surface exposure of PS. Bhatia et al. reported that ceramide promoted the exocytosis of Weibel-Palade bodies. Thus, reduced cellular ceramide may offer an additional, distinct mechanism to blunt thrombosis through a reduced capacity to secrete von Willebrand factor (vWF).

In contrast to the changes observed in SM and CER, the reduction in PS was observed at the level of the whole cell and purified plasma membrane. In most cases, factor VIIa/TF activity and prothrombinase activities were reduced in proportion to PS levels (Tables 1 and 2, Figures 3 and 4), and on mevalonate addition prothrombinase activity recovered to the same extent as PS concentrations (Table 1 and Figure 4). This finding strongly suggests that PS exposure is controlled by the membrane concentration of PS. We cannot rule out, however, that changes in the expression of phospholipid scrambling or translocating enzymes also play a role in the diminished exposure of PS. Changes in membrane PS exposure may also impact other cellular processes, namely the recognition and engulfment of senescent cells, vesicular fusion with the plasma membrane, and membrane blebbing to form microparticles. These questions are the focus of ongoing studies.

Clinical evidence has shown that statins markedly reduce the risk of venous thromboembolism. Our study suggests that the effect of statins on membrane content are responsible for this global anticoagulant effect. It remains to be demonstrated, however, that the phospholipid alterations we observed are sufficient to produce a membrane with enhanced anticoagulant properties. Various anticoagulant systems (TFPI, antithrombin III, and Protein C) must be unaltered or potentiated by the membrane changes displayed in this study to produce a net anticoagulant effect. TFPI expression is not altered by statin treatment (data not shown) and is not altered by its lipid environment. Antithrombin III activity is generally not regulated by the membrane lipid but rather by glycosaminoglycans present on the cell surface. Activated protein C (APC) activity is acutely sensitive to the membrane environment, but it has been shown that lipid alterations impact APC activity and prothrombinase activity in different ways. Smirnov et al. showed that lupus anticoagulant immunoglobulin inhibited APC activity far greater than prothrombinase activity on phospholipid vesicles. In light of these considerations, it is plausible that statins have a specific and isolated impact on the procoagulant protease cascade. The extent to which this mechanism explains the anticoagulant properties of statins remains to be determined.

The culture conditions for the present study using both immortalized and primary cells were carefully optimized to provide as much physiological relevance as possible. Other studies have used short-term exposure of cells to cytotoxic concentrations of statins (2 to 100 μmol/L), Conclusions from these studies are limited by undefined effects on cell viability. Our conditions used concentrations of statins that would realistically be achieved in the circulation, but on the other hand used serum containing far less cholesterol than is found in human serum. However, our data indicate that the changes induced by atorvastatin are not modulated by cholesterol but rather by mevalonate-derived intermediates. The identification of these metabolic intermediates and their change in concentration after statin administration will be necessary to validate our findings in vivo.

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Disclosures

None.

References


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Supplemental Figure 1: Prothrombinase activity is proportional to exposed phosphatidylserine. The prothrombinase complex was reconstituted in the presence of a monolayer of EA.hy926 cells in a 96 well culture dish. Reactions were initiated by the addition of factor V following a 15 minute preincubation with cell buffer or cell buffer containing the indicated concentrations of annexin V. Activity was followed continuously for 60 minutes and linearized using the first derivative of the non-linear increase at 405 nm. Data are means±SD for 4 experiments. Inset: Prothrombinase activity and WST-1 oxidation were monitored in replicate wells to assess the dependence of activity on cell number. Prothrombinase rates ($A_{405}$/$\min^2$) were proportional to cell number as indicated by WST-1 absorbance.