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Inhibition of the Thrombogenic and Inflammatory Properties of Antiphospholipid Antibodies by Fluvastatin in an In Vivo Animal Model

Dardo E. Ferrara,[1] Xiaowei Liu,[1] Ricardo G. Espinola,[1] Pier Luigi Meroni,[2] Imad Abukhalaf,[1] E. Nigel Harris,[1] and Silvia S. Pierangeli[1]

Objective. **Antiphospholipid antibodies (aPL) have thrombogenic properties in vivo, through their interactions with soluble coagulation factors and their ability to modulate the functions of cells involved in coagulation homeostasis. These antibodies have also been shown to enhance the adhesion of leukocytes to endothelial cells (ECs) in vivo. New lipophilic statins such as fluvastatin have antiinflammatory and antithrombogenic effects. This study uses an in vivo mouse model to investigate whether fluvastatin has an effect on decreasing both the adhesion of leukocytes to ECs and the thrombus formation induced by aPL.**

Methods. **Two groups of CD-1 male mice, each comprising ~18 mice, were fed either normal saline solution or 15 mg/kg fluvastatin for 15 days. Each of the 2 groups was further subdivided to receive either purified IgG from patients with the antiphospholipid syndrome (IgG-APS) or normal IgG from healthy subjects. Analysis of thrombus dynamics was performed in treated and control mice, using a standardized thrombogenic injury procedure, and the area (size) of the thrombus was measured. Adhesion of leukocytes to ECs was analyzed with a microcirculation model of exposed cremaster muscle. Baseline and posttreatment soluble intercellular adhesion molecule 1 (sICAM-1) levels were determined by enzyme-linked immunosorbent assay.**

Results. **IgG-APS mice treated with fluvastatin showed significantly smaller thrombi, a reduced number of adherent leukocytes, and decreased levels of sICAM-1 compared with IgG-APS animals treated with placebo.**

Conclusion. **These findings indicate that fluvastatin significantly diminishes aPL-mediated thrombosis and EC activation in vivo. These results may have important implications for the design of new treatment strategies aimed at preventing recurrent thrombosis in patients with APS.**

Recurrent venous and/or arterial thrombosis, pregnancy losses, and the presence of either medium or high levels of anticardiolipin (aCL) antibodies or positivity on the lupus anticoagulant test are the most significant features of the antiphospholipid syndrome (APS) (1,2). Experimental evidence shows that antiphospholipid antibodies (aPL) not only are markers of the disease, but also may play a causative role in the development of vascular thrombosis and pregnancy morbidity (3–8). Nonetheless, the pathogenic mechanisms of aPL seem to be heterogeneous and far from being completely understood (9). Among the mechanisms suggested to explain the prothrombotic activity of aPL are the direct inhibition of the activated protein C pathway (10), abnormalities in platelet function (11,12), up-regulation of the tissue factor pathway (13), and activation of endothelial cells (ECs) (14).

In particular, recent studies have shown that aPL or anti–β_2-glycoprotein I (β_2GPI) antibodies induce proadhesive, proinflammatory, and procoagulant molecules that provide a persuasive explanation for induction of thrombosis in APS (15). One marker for expression of EC activation is adherence of leukocytes to the vascular endothelium. This adherence is associated with the

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[1]Dardo E. Ferrara, MD, Xiaowei Liu, MD, Ricardo G. Espinola, MD, Imad Abukhalaf, PhD, E. Nigel Harris, MD, DM, Silvia S. Pierangeli, PhD: Morehouse School of Medicine, Atlanta, Georgia; [2]Pier Luigi Meroni, MD: University of Milan, IRCCS Istituto Auxologico Italiano, Milan, Italy.

Address correspondence and reprint requests to Silvia S. Pierangeli, PhD, Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, 720 Westview Dr. SW, Atlanta, GA 30310-1495. E-mail: pierans@msm.edu.

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expression of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1, and selectins (15). EC activation by aPL may result not only in expression of adhesion molecules, but also in production of cytokines and expression of tissue factor (13).

The 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors, or statins, are potent inhibitors of cholesterol synthesis. Clinical trials of statin therapy have demonstrated beneficial effects in primary and secondary prevention of coronary heart disease as well as ischemic stroke (16–21). Their beneficial effects are only partially explained by their ability to lower cholesterol levels. Statins have also been shown to modify the function of ECs, smooth muscle cells, platelets, and monocytes/macrophages (22). Their effects include decreasing the expression of adhesion molecules in monocytes and leukocyte–endothelial interactions (23–26), inhibiting platelet function (27), inhibiting tissue factor expression by mononuclear cells (28,29), down-regulating inflammatory cytokines in ECs (30), increasing fibrinolytic activity (31,32), and immunomodulation by decreasing the expression of class II major histocompatibility complex antigen (33).

Little is known about the effectiveness of statins in preventing the development of deep vein thrombosis (DVT). One recent observational study has addressed this question by showing that statins may play a role in the prevention of DVT (34,35). In addition, Meroni and colleagues have shown that fluvastatin and simvastatin prevent expression of adhesion molecules and production of interleukin-6 by ECs. They reported that statins might act through inhibition of NF-κB activation (36).

The aim of this study was to determine whether peroral administration of fluvastatin to mice injected with purified IgG-aPL antibodies from patients with APS (IgG-APS) might result in reduced adherence of leukocytes to ECs and reduced formation of venous thrombus. To test this hypothesis, we used a previously devised mouse model of APS in which human polyclonal and monoclonal aCL antibodies have been demonstrated to increase the thrombus size induced by a standardized injury (4–6). We also used a microcirculation model of mouse cremaster muscle to study the effects of aPL on monocyte adherence to ECs (37). A secondary end point of this study was assessment of the ability of IgG-APS to increase levels of a marker of EC activation, namely soluble ICAM-1 (sICAM-1), and whether fluvastatin would inhibit levels of this marker.

Table 1. Experimental design of the in vivo experiments

Group	No. of mice	Treatment*
A	9	Saline solution + IgG-APS
B	10	Fluvastatin + IgG-APS
C	8	Saline solution + IgG-NHS
D	8	Fluvastatin + IgG-NHS

* IgG-APS = purified IgG from patients with the antiphospholipid syndrome; IgG-NHS = normal IgG from healthy subjects.

MATERIALS AND METHODS

Animals. Male CD-1 mice weighing ~30 gm were purchased from Charles River Laboratories (Wilmington, MN). The animals were housed in the animal care facilities at Morehouse School of Medicine in Atlanta and handled by trained personnel according to Institutional Animal Care and Use Committee guidelines under the supervision of veterinarians.

Reagents. Fluvastatin sodium (powder form) was kindly provided by Novartis Pharma (Basel, Switzerland) through one of the authors (PLM). This drug, as sodium salt, was dissolved in sterile normal saline immediately before use and administered at a dose of 15 mg/kg once daily. This treatment level represents plasma drug levels of ~2 times the mean drug concentration in human plasma after a 40 mg oral dose.

Isolation of IgG from APS patients and normal subjects. The aPL antibodies (IgG-APS) were obtained from the sera of an APS patient and purified with the use of DEAE ion-exchange chromatography as described elsewhere (38). As control, aCL- and lupus anticoagulant–negative, pooled, normal plasma from healthy subjects was used as a source of normal IgG (IgG-NHS). Similarly, purification was done by using DEAE Sepharose. The aCL activity, expressed in IgG phospholipid (GPL) units, was measured using a standardized enzyme-linked immunosorbent assay (ELISA). The anti-β_2GPI activity was determined by ELISA, utilizing a commercial kit (Quanta Lite; Inova Diagnostics, La Jolla, CA).

Experimental design. Two groups of ~18 male CD-1 mice were assigned to be perorally fed during 15 days with either normal saline or fluvastatin (15 mg/kg body weight) diluted in saline solution. Each of these 2 initial groups was equally divided in 2 further subsets to receive either intraperitoneal injection with an IgG-APS preparation (1 mg antibody per injection in 1 ml of sterile saline solution) or the IgG-NHS control at feeding days 13 and 15. The aCL activity of the IgG-APS preparation was 95 GPL units when bovine serum was used as the source of β_2GPI and 105 GPL units when the murine serum was used as the source of β_2GPI in the diluent and blocking solutions, indicating that the preparation recognizes murine β_2GPI. The anti-β_2GPI activity was 125 standard IgG units/ml, as detected by ELISA. In this way, 4 groups of mice were studied, and each group consisted of 8–10 animals (Table 1).

Two surgical procedures (described below) were performed in the same mouse at 72 hours after the first injection, corresponding to day 16 from the first day of feeding. Samples of blood were collected before and after the drug treatment and antibody injections, to determine the levels of serum total

cholesterol and sICAM-1. The aCL ELISA test was also performed to determine the titers of human aCL antibody in the sera of the mice.

In each mouse in each of the 4 groups, the dynamics of thrombus formation in the exposed femoral vein and leukocyte adhesion to ECs in the exposed cremaster muscle were determined in accordance with previously described methods (15,37,39). The person in charge of injecting mice with IgG-APS or IgG-NHS (RGE) was blinded to the treatment given perorally to each animal, and the microvascular surgeon (XL) was blinded to all of the substances administered.

Determination of fluvastatin levels in the serum of mice. Since oral fluvastatin is 98% absorbed (40), prior studies using the same feeding method did not attempt to detect the presence of the drug in the blood of the animals (41–44). However, in order to prove that fluvastatin is absorbed through the gastrointestinal tract, we validated our peroral feeding method by determining the presence of the drug in the blood of another group of mice. To achieve this goal, we used high-performance liquid chromatography as previously described (45).

Surgical procedures. *Analysis of thrombus dynamics.* The analysis of thrombus dynamics in a mouse model has been described previously (4–6,46). Briefly, mice were anesthetized at 72 hours after the first injection with IgG-APS or IgG-NHS. The right femoral vein was then exposed and a standardized thrombogenic injury was produced with a pinch injury. Clot formation and dissolution in the transilluminated vein were visualized with a microscope equipped with a closed-circuit video system (including a color monitor and a recorder). Thrombus size (in square micrometers) was measured when the thrombus reached its maximum size, by digitizing the image and tracing the outer margin of the thrombus. The mean thrombus area and the mean times for formation and for disappearance of the thrombus, as well as total duration of thrombus formation were then computed for each group of animals (4–6,37).

Analysis of EC activation in the microcirculation of the exposed cremaster muscle in mice. The ability of aPL antibodies (IgG-APS) to activate ECs in vivo was assessed by direct visualization and quantification of leukocytes (white blood cells [WBCs]) adhering to ECs in the microcirculation of the exposed cremaster muscle of mice, as described elsewhere (15,37,39). After a stabilization period of 30 minutes, the number of adhering WBCs that remained stationary for a period of 30 seconds (sticking) within 5 different postcapillary venules (diameter of 25–35 μm) was determined. The mean numbers of WBCs were calculated and compared among all treated and control groups.

Measurements of cholesterol and sICAM-1 levels in serum. The concentration of total serum cholesterol was enzymatically measured in the blood samples collected on the day of surgery, by using a commercial kit (Sigma, St. Louis, MO). Soluble ICAM-1 levels were measured using an in vitro ELISA commercial kit (Endogen Mouse Soluble ICAM-1 ELISA) that does not cross-react with human sICAM-1. Blood samples were drawn 24 hours after the last challenge with aPL, corresponding to the moment when the expression of ICAM-1 usually reaches stable values (47).

Statistical analysis. One-way analysis of variance was used to compare the mean thrombus sizes, times of thrombus duration, and adherence by WBC numbers for the 4 groups. The number of animals needed per group was determined by power analysis. Statistical significance was considered to be achieved at P values less than or equal to 0.05.

RESULTS

Levels of aCL. Table 2 compares the aCL levels between the 2 IgG-APS groups of mice treated with fluvastatin or placebo. High levels of aCL were achieved in both of these groups, and no statistically significant difference was observed. Mice injected with IgG-NHS were negative for aCL antibodies.

Levels of fluvastatin in treated and control mice. The mean ($\pm$SD) levels of fluvastatin in the serum of mice fed with the drug was 1,268.6 $\pm$ 560 ng/ml at 2 hours after ingestion of the drug. Mice fed with placebo had undetectable levels of fluvastatin in their serum, as determined by chromatography.

In vivo experiments. *Effect of fluvastatin on the dynamics of thrombus formation.* Placebo-treated animals injected with IgG-APS (group A) produced significantly larger thrombi than did placebo-treated animals injected with IgG-NHS (group C), indicating that the IgG-APS enhanced thrombus formation. Similar results have been obtained previously (4–6) (Figure 1). The thrombus size in animals injected with IgG-APS and treated with fluvastatin (group B) was significantly reduced compared with that in animals injected with IgG-APS and treated with placebo (group A). There was no significant difference in thrombus size between the fluvastatin-treated and the placebo-treated mice injected with normal IgG (groups D and C, respectively) (Figure 1). In addition, values obtained by treatment of IgG-APS mice with fluvastatin (group B) were not significantly different from those obtained in either the placebo-treated or fluvastatin-treated groups of IgG-NHS mice (groups C and D, respectively) (Figure 1). These results indicate that fluvastatin at a dosage of 15 mg/kg/day during 15 days may completely prevent the enhanced thrombus formation associated with aCL antibodies.

Table 2. Anticardiolipin (aCL) levels in the serum of mice injected with IgG-APS*

Treatment	No. of mice	Mean $\pm$ SD aCL level, GPL units
IgG-APS + fluvastatin (group B)	10	101.7 $\pm$ 26.4
IgG-APS + saline (group A)	9	111.4 $\pm$ 22.4

* IgG-APS = purified IgG from patients with the antiphospholipid syndrome; GPL = IgG phospholipid.

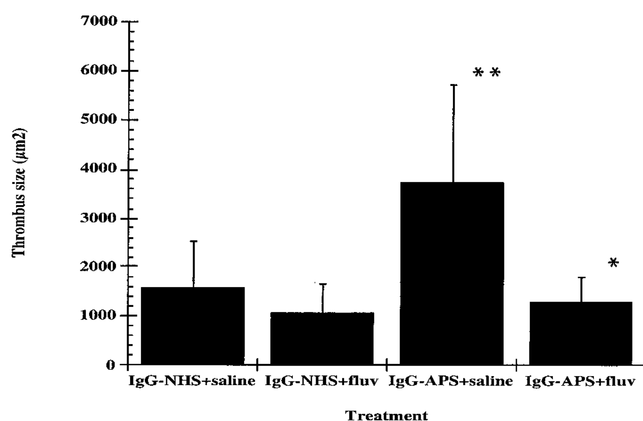

Figure 1. Effects of fluvastatin (fluv) on thrombus formation in mice injected with antiphospholipid antibodies. The size of an induced thrombus was measured in the femoral vein, as described in Materials and Methods, among mice administered normal IgG from healthy subjects (IgG-NHS) and treated with saline (group C in Table 1) or fluvastatin (15 mg/kg/15 days) (group D in Table 1), and among mice administered purified IgG from patients with the antiphospholipid syndrome (IgG-APS) and treated with saline (group A in Table 1) or fluvastatin (15 mg/kg/15 days) (group B in Table 1). Bars show the mean and SD. * = statistically significant difference ($P \leq 0.05$) from the mean value in group A. ** = statistically significant difference ($P \leq 0.05$) from the mean value in group C.

Effect of fluvastatin on adhesion of leukocytes to ECs. As shown in Figure 2, the adhesion of leukocytes to ECs in animals injected with IgG-APS and fed with saline solution (placebo group A) was significantly increased when compared with the number of leukocytes adhering to ECs in mice given IgG-NHS and saline (group C) (mean ± SD 8.8 ± 1.7 versus 4.2 ± 1.3). Similar results have been shown previously (15,37). Fluvastatin significantly reduced the aPL-induced increase in the number of leukocytes adhering to endothelium, from a mean ± SD 8.8 ± 1.7 to 4.4 ± 1.1 (Figure 2).

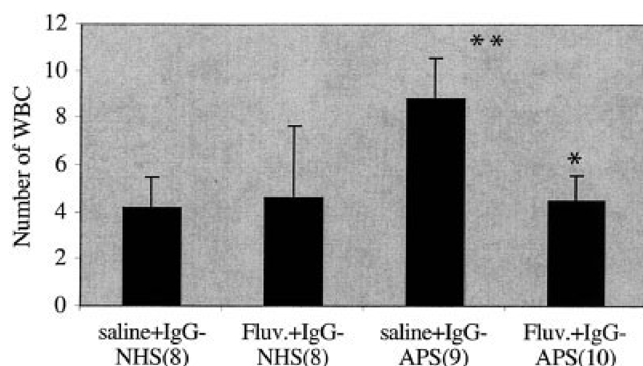

Figure 2. Effects of fluvastatin on adhesion of leukocytes to endothelial cells in mice treated with antiphospholipid antibodies. The number of leukocytes (white blood cells [WBC]) adhering to the endothelium of mouse cremaster muscle postcapillary venules was determined in mice with IgG-NHS treated with saline (group C in Table 1) or fluvastatin (15 mg/kg/15 days) (group D in Table 1), and in mice with IgG-APS and treated with saline (group A in Table 1) or fluvastatin (15 mg/kg/15 days) (group B in Table 1), as described in Materials and Methods. Bars show the mean and SD. * = statistically significant difference ($P \leq 0.05$) from the mean value in group A. ** = statistically significant difference ($P \leq 0.05$) from the mean value in group C. See Figure 1 for other definitions.

Table 3. Cholesterol levels in the serum of mice treated with saline solution or with fluvastatin

Treatment	No. of mice	Mean ± SD cholesterol level, mg/dl
Saline solution for 15 days	11	121.1 ± 20.4
Fluvastatin (15 mg/kg) for 15 days	15	120.4 ± 29.3

Serum cholesterol levels. Table 3 summarizes the total serum cholesterol concentrations in mice treated with fluvastatin and those treated with saline (placebo). The fluvastatin treatment did not alter the serum cholesterol levels.

Effect of fluvastatin on sICAM-1 serum levels. The mean serum concentrations of sICAM-1 significantly increased in the IgG-APS group (group A) com-

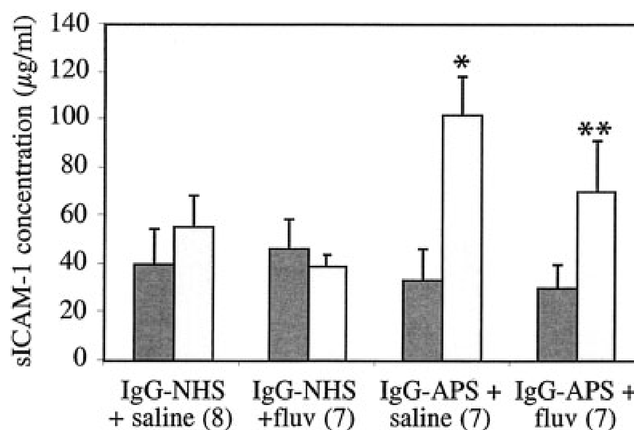

Figure 3. Effects of fluvastatin on levels of soluble intercellular adhesion molecule 1 (sICAM-1) in mice treated with antiphospholipid antibodies. The serum levels of sICAM-1 were measured in mice with IgG-NHS treated with saline (group C in Table 1) or fluvastatin (15 mg/kg/15 days) (group D in Table 1), and in mice with IgG-APS treated with saline (group A in Table 1) or fluvastatin (15 mg/kg/15 days) (group B in Table 1). The mean and SD values were determined at baseline (shaded bar) and on day 16 after treatment (open bar), as described in Materials and Methods. * = statistically significant difference ($P \leq 0.05$) from the mean value in baseline conditions; ** = statistically significant difference ($P \leq 0.05$) from the mean value in group A after 16 days. See Figure 1 for other definitions.

pared with the group that received IgG-NHS (group C) after these animals were fed with saline for 15 days (increasing from 33.2 $\pm$ 12.6 μg/ml to 101.8 $\pm$ 16.4 μg/ml compared with an increase from 39.4 $\pm$ 15.4 μg/ml to 55.1 $\pm$13.7 μg/ml, respectively, from baseline to day 16) (Figure 3). Treatment with fluvastatin partially, but significantly, reduced the concentration of this inflammatory molecule by 30% in the IgG-APS group (group B) (Figure 3). Fluvastatin did not significantly affect the serum levels of sICAM-1 in mice injected with IgG-NHS (group D).

DISCUSSION

Monoclonal and polyclonal aPL antibodies have previously been demonstrated to have thrombogenic properties in mice (6,37,46,48). Several studies have demonstrated that aPL activate ECs in vitro, suggesting a mechanism by which these antibodies exert procoagulant properties (15,49,50). Utilizing a mouse model, our group has previously demonstrated that aPL antibodies increase leukocyte adhesion to ECs of cremasteric muscle postcapillary venules, suggesting that these antibodies induce activation of ECs in vivo and that these effects correlate with enhanced thrombus formation (37). This was confirmed by subsequent studies showing that the effect of aPL on leukocyte adhesion was abrogated in ICAM and P-selectin knockout mice (15).

In this investigation, fluvastatin was found to significantly blunt the thrombogenic response (thrombus size) and inflammatory response (adhesion of leukocytes to postcapillary venules and levels of sICAM-1) elicited by injecting mice with IgG-APS. This confirms the observation of Meroni et al, who recently demonstrated with the use of in vitro experiments that fluvastatin modulates the expression of adhesion molecules and cytokine production by ECs activated by aPL (36).

There are several reports suggesting that statins such as simvastatin and fluvastatin may exert an antithrombotic effect. One possible mechanism of this effect includes the inhibition of tissue factor expression (51,52). In a recent report, thrombogenicity of the arterial wall of hypercholesterolemic rabbits was decreased by fluvastatin and this was accompanied by a reduction in tissue factor expression and decrease in NF-κB activation in the aortic arch (53). The authors concluded that fluvastatin treatment reduced thrombogenesis by inhibiting tissue factor synthesis. The fact that aPL antibodies have recently been shown to up-regulate tissue factor expression, and the observation that lipophilic statins (fluvastatin, simvastatin) suppress tissue factor expression, offer a possible explanation for the observations of the present study, namely, that the inhibitory effects of fluvastatin on aPL-induced thrombus formation seem stronger when compared with similar effects of the drug on the aPL-induced adhesion of leukocytes to endothelium.

In this study, the effects of fluvastatin on thrombus formation and EC activation were independent of the cholesterol-lowering effect of the drug. This observation is not surprising. The enzyme HMG-CoA reductase is responsible for the conversion of HMG-CoA to mevalonate. Since mevalonic acid is the precursor not only of cholesterol, but also of many nonsteroidal isoprenoid compounds critical for several cellular processes of eukaryotic cells, inhibition of the mevalonate pathway by statins has pleiotropic effects (52). Many of these pleiotropic effects of statins are mediated by their ability to block the synthesis of important isoprenoid intermediates, which serve as lipid attachments for a variety of intracellular signaling molecules and have been shown to occur in the absence of a reduction of the cholesterol levels (54–56). In particular, the inhibition of small GTP-binding proteins, Rho, Ras, and Rac, whose proper membrane localization and function are dependent on isoprenylation, may play an important role in mediating the direct cellular effects of statins on the vascular wall (22,52–56).

Based on available data, Fenton et al postulated that HMG-CoA reductase inhibitors may decrease tissue factor expression and down-regulate cell signaling following thrombin activation of protease-activated receptor 1, thus exerting an antithrombotic effect (56). These investigators proposed that statins may constitute a new class of antithrombotic drugs that are possibly effective in patients with a prothrombotic tendency (56). Statins have also been shown to decrease factor VII coagulant activity in patients with hyperlipidemia (57) and to enhance factor Va inactivation by activated protein C (58). These latter effects may also inhibit thrombus formation.

Most of the inhibitory effects of statins have been postulated to occur in the arterial circulation, but their actions in the venous circulation are unknown. It is possible that their effects are comparable in both arterial and venous ECs. Of relevance is a retrospective subgroup analysis by investigators of the Heart Estrogen Replacement Study showing that use of statins was associated with a 50% reduction in the risk of venous thromboembolism (34). More recently, a Canadian retrospective cohort study showed that use of these drugs was associated with a 22% reduction in the relative risk

of DVT (35). Because these data do not come from randomized controlled trials, conclusions should be viewed with caution.

The findings of the present study support proposals that fluvastatin suppresses thrombus formation and IgG-APS–induced EC activation in the venous circulation. These effects were shown not to be related to lowered cholesterol levels. Interestingly, we showed that fluvastatin was effective at a dose that produces plasma concentrations close to the levels occurring in subjects taking this drug at therapeutic doses. In our study, and in order to validate the peroral feeding method, we examined the levels of fluvastatin in the blood of mice after 2 hours of ingestion of the drug. The levels of fluvastatin obtained, although higher than the values reported in humans receiving typical doses (52), are comparable with the levels obtained in humans after administration of high doses of the drug (45). These differences may be due to several factors, including differences in absorption, kinetics, and metabolism of the drug in rodents, and differences in food ingestion, among other factors. Importantly, our data confirm that fluvastatin was effectively administered and absorbed in our experiments in mice, after the animals were fed concentrations of the drug similar to the concentrations used to reduce cholesterol levels in humans.

To our knowledge, this is the first study to demonstrate an in vivo inhibitory effect of statins on thrombus formation induced by high titers of aCL antibodies. It is also the first to utilize this model to demonstrate that statins may prevent venous thrombosis. The exact mechanisms by which fluvastatin reverses the thrombogenic properties of aCL antibodies have yet to be fully elucidated.

One possible mechanism is suppression of adhesion molecules expressed on the surface of ECs by aPL antibodies, as our results demonstrated by the reduction in leukocyte adhesion to capillary ECs. In addition, we demonstrated that IgG-APS increased concentrations of sICAM-1, but pretreatment with fluvastatin suppressed this increase. Previous studies have demonstrated that increases in the circulating concentrations of these molecules are proportional to the increases in their cell membrane expression (59–63). Furthermore, the soluble phase of this molecule seems to have biologic activity. Soluble ICAM-1 has been shown to interact with the leukocyte β_2 integrin (LFA-1), and it has been proposed as a useful marker of the inflammatory response (64). Closely related to this mechanism, statins have been shown to suppress the inflammatory response by binding directly to a regulatory site of the LFA-1 β_2 integrin, which serves as a major counterreceptor for ICAM-1 on leukocytes (24).

It is conceivable that statins may be beneficial in a variety of circumstances in patients with APS. They might even replace warfarin in prevention of recurrent arterial and venous thrombosis (65,66), thus eliminating the risk of the hemorrhagic complications associated with warfarin and enabling better lifestyles in these patients. Statins may also serve as an alternative treatment in APS patients who experience thrombosis despite adequate anticoagulation with warfarin, or in those with thrombocytopenia, in whom warfarin is contraindicated. Finally, statins would be an appealing alternative to warfarin as prophylactic therapy in patients with high levels of aCL antibodies and without a history of thrombosis. Although the latter group of patients may be at enhanced risk of thrombosis (67), many physicians believe the risk of prolonged warfarin therapy may outweigh any potential benefits. Unfortunately, statins are teratogenic, producing fetal skeletal abnormalities, and therefore their use in pregnancy is contraindicated (40). Thus, the prospective role of fluvastatin in the management of patients with APS will need to be further defined in clinical studies.

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