Inhibition of Platelet-Rich Arterial Thrombus In Vivo
Acute Antithrombotic Effect of Intravenous HMG-CoA Reductase Therapy

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Objective—To test the hypothesis that statins will acutely inhibit platelet thrombus formation, intravenous lovastatin was assessed in our well-characterized porcine carotid injury model.

Methods and Results—The first carotid artery was crush-injured and harvested after 30 minutes. Pigs then received intravenous lovastatin (100 μg/kg bolus + 100 μg/kg/h infusion, n=6) or saline (n=11) before injury of the second carotid artery. Thrombus size was quantified by scintillation detection of autologous 111In-platelets. Sequential carotid injury produced a thrombus more than 50% greater in volume in the second (3149±2053×10^6/cm^2) relative to the first injured artery (2081±1552×10^6/cm^2, P=0.04) in control pigs. This augmentation was inhibited by intravenous lovastatin which acutely reduced platelet deposition (944±246×10^6/cm^2) relative to saline control (P=0.02). Flow chamber closure times increased on average by 2.45-fold in response to whole blood lovastatin incubation. Lovastatin (P<0.05) and simvastatin (P<0.05) reduced platelet dense granule secretion in vitro.

Conclusions—Sequential arterial injury augments the thrombotic response suggesting that the propensity for arterial thrombosis is at least partially acquired. This thrombotic augmentation can be acutely attenuated by intravenous lovastatin which may result from a pleiotropic impact on platelet function. These results appear to be a class effect of 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors. (Arterioscler Thromb Vasc Biol. 2009;29:1271-1276.)

Key Words: thrombosis ■ platelets ■ statins ■ pleiotropic effects ■ HMG-CoA reductase inhibitors

Statins inhibit cholesterol synthesis in the liver by blocking the conversion of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) to mevalonate, the rate-limiting step in the mevalonate pathway. Lowering of LDL plasma levels has a long-term favorable impact on both the primary and secondary prevention of cardiovascular events including myocardial infarction, stroke, cardiovascular, and all cause mortality. The reduction of total and LDL cholesterol with these agents may both improve luminal patency and stabilize plaque in atherosclerotic diseased vessels, benefits which are thought to occur over months to years of treatment.

However, recent clinical observations indicate that statins demonstrate beneficial effects on cardiovascular complication rates and survival very quickly and independently of their cholesterol lowering effect. Data from large randomized trials in acute coronary syndromes for example have shown improved major adverse cardiac events including mortality as soon as 1 month after statin initiation. Pretreatment with statins one week before elective angioplasty reduced procedure-related myocardial infarction by more than 80%. In patients with non-ST segment elevation myocardial infarction, pretreatment with statins for 12 hours before coronary intervention reduced the incidence of cardiac events by 70%.

Published results from the National Registry of Myocardial Infarction 4 database of more than 170 000 patients showed that statin treatment within the first 24 hours of an acute MI markedly decreased mortality. Furthermore, discontinuation of statin treatment in these patients was associated with increased mortality. Attributed to the pleiotropic effects of statins, these effects may be explained by statin inhibition of isoprenoid synthesis, key elements in cell signaling. In this way, statins may exert a favorable effects on inflammation, the endothelium, and the coagulation cascade. Although the long-term impact of lipid lowering by statin therapy has been well demonstrated, the impact and temporal onset of the pleiotropic benefits on prothrombotic pathways are less well established. To this end, we tested the hypothesis that HMG-CoA reductase inhibition by intravenous statin therapy will promptly reduce platelet thrombus formation following carotid crush injury in pigs.

Materials and Methods

Animals and Reagents

Four-month-old, pre-estrus, female pigs (average weight 40 kg) of the Babcock 4-way cross stock (a mixture of Landrace, Yorkshire, Yorkshire, and Large White breeds) were used for these studies. Animals and procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, National Institutes of Health, Bethesda, Md. Four-month-old, pre-estrus, female pigs (average weight 40 kg) of the Babcock 4-way cross stock (a mixture of Landrace, Yorkshire, Yorkshire, and Large White breeds) were used for these studies. Animals and procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, National Institutes of Health, Bethesda, Md.

Received August 8, 2008; revision accepted June 20, 2009.
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© 2009 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.109.190884

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Hampshire, and Duroc breeds) were purchased from local vendors through the Mayo Clinic’s section of Veterinary Medicine, housed at the Mayo Institute Hills Facility, and fed a normal chow diet. The study was approved by the Mayo Clinic Animal Care and Use Committee and conformed to the National Institutes of Health and United States Department of Agriculture guidelines.

Mevinolin (Lovastatin) and simvastatin were purchased from Sigma Aldrich Chemical Co and reconstituted (10 mg/mL) in ethanol and then diluted with buffered saline before use.

**Induction of Thrombosis**

Anesthesia of pigs, autologous 111In-platelet labeling, and carotid arterial crush injuries were performed as described previously. A randomly chosen carotid artery was serially injured by 6 serial hemostat crushes of 3-second duration, interspersed with a 3-second rest period, with each subsequent injury visually abutting the prior injury site. The thrombus was then allowed to propagate for 30 minutes, after which the vessel was ligated proximally and distally to the injury site and harvested. Pigs then received either intravenous Lovastatin (100 μg/kg bolus + 100 μg/kg/h infusion, n = 6) or saline (n = 11). Thirty minutes after drug initiation, the contralateral artery was injured in the same manner and harvested after 30 minutes. At the end of each preparation, both injured arterial segments were assayed for 111In content in a scintillation counter.

Ear bleeding times and activated whole blood clotting times were performed as previously described.

**Lovastatin Dosing**

The dose of lovastatin (MW 400) was based on our preliminary findings that 200 nmol/L mevinolin incubated with platelet rich plasma for 90 minutes substantially inhibited platelet function. Assuming an average plasma volume of 2 L (body weight in kg × 0.05), each animal was given 200 μg/kg (bolus plus infusion) which corresponds to 100 ng/mL final plasma concentration. At the time of injury, the circulating concentration was approximately 190 nmol/L and approximately 250 nmol/L at vessel harvest. Whereas each pig weighed 40 kg on average, the total dose per animal was 8 mg lovastatin.

**Platelet Reactivity Test Assay**

This assay is based on the premise that platelets flowing under high shear conditions will adhere to foreign surfaces within the flow pathway promoting platelet activation and aggregation. Forty microliters of a whole blood sample collected into r-hirudin (20 nmol/L) and tick anticoagulant peptide (10 nmol/L) was incubated with lovastatin (200 nmol/L) or saline for 30 minutes at room temperature. Samples were then subjected to reciprocating shear forces (peak shear rate: 1600 seconds⁻¹) through a microcapillary bed. A copper coil imbedded within the center of the 50-μm flow channel served as a surface to which platelets adhere. Occlusion of the flow path by platelet thrombus triggers a pressure sensor and provides a “time to occlusion” end point. Whereas the capillary channel itself is composed of clear plastic, the resultant white platelet thrombus can be identified by light microscopy.

**Platelet Deposition**

Platelet deposition after serial carotid arterial injury. When performed in series, injury to the second carotid artery results in more than 50% augmentation in platelet deposition relative to the first carotid artery in control pigs (n = 11). This augmented thrombotic response is attenuated in pigs treated with intravenous lovastatin (n = 6).

**Statistics**

All values are presented as mean ± SD. Wilcoxon signed rank test was used to test treatment effects of statin therapy between groups in vivo. Paired Student t test was used to compare treatment effects of antithrombotic agents before and after chamber perfusion and to compare hemoglobin, hematocrit, and platelet counts before and after each surgery. Statistical significance was set at P < 0.05.

**Results**

When performed serially in control animals, platelet-rich thrombus formation increased on average by 55% in the second relative to the first injury (Figure 1 and 2). The mean platelet deposition in the second carotid artery (3149 ± 2053 × 10⁶/cm²) was significantly greater than the first carotid artery (2081 ± 1552 × 10⁶/cm²; P = 0.04) in control pigs. For the control animals (n = 11), the carotid arterial flow rates before the first arterial injury averaged 130 ± 35 mL/min (range 100 to 223 mL/min). Flow rates before the second injury averaged 158 ± 87 mL/min (range 50 to 300 mL/min). Although flow rates were higher in the second artery relative to the first, this difference was not significant (P = 0.27). To determine whether duration of anesthetic exposure impacts platelet deposition, the time between induction and initial
carotid injury was lengthened by up to 60 minutes (n=3). There was no significant difference in platelet deposition comparing longer to shorter anesthesia times for either the first (P=0.27) or second (P=0.65) carotid injury. To evaluate the influence of arterial crush injury on measures of peripheral blood platelet activity, platelet dense granule secretion was compared at baseline and after the second carotid injury. On average, a 26% (± 5%) reduction in platelet secretion was observed, yet this did not reach statistical significance (P=0.16).

The administration of intravenous lovastatin attenuated platelet thrombus formation in the second injured artery (944±246×10⁶/cm²) compared to the first carotid injury (1672±1140×10⁶/cm²; Figures 1 and 2).

To determine whether these antithrombotic effects could be attributed to an antiplatelet mechanism, dense granule secretion was assessed for platelets incubated with lovastatin at 2 concentrations in vitro. After a 30-minute incubation, the dense granule ATP release response to stimulation by porcine thrombin declined by 10% (50 nmol/L lovastatin) and 22% (200 nmol/L lovastatin) dependent on the lovastatin dose (Figure 3; P<0.05 for each comparison). Dense granule secretion was also reduced after convulxin stimulation by 23% to 27%, yet this did not reach statistical significance (P=0.06 for lovastatin 200 nmol/L). Thrombin-induced platelet dense granule secretion correlated with convulxin dense granule secretion across the dose range (P<0.05; data not shown).

To determine the timing of maximal platelet inhibition and to assess whether the results of platelet inhibition were related to lovastatin specifically or statins in general, dense granule secretions were compared between lovastatin (200 nmol/L) and simvastatin (200 nmol/L) over time. After stimulation with convulxin, similar reduction of porcine platelet dense body granule secretion was noted for lovastatin (33%) and simvastatin (25%) compared to saline (Figure 4A; P<0.05).

To determine whether statin induced platelet inhibition occurred as a result of impaired signaling through alteration of protein prenylation, platelets were preincubation with farnesyl pyrophosphate (FPP) and either lovastatin, simvastatin, or saline. An appreciable improvement in platelet secretion with the addition of FPP to statin-treated platelets was not observed (Figure 4B). A nonsignificant 40% increase in the platelet ATP secretion was, however, observed in platelets incubated with FPP and saline, suggesting both that FPP is an important mediator of this functionality and that it can be rapidly absorbed into these cells.

In keeping with both the in vivo and in vitro antithrombotic response, the platelet reactivity test closure times were significantly prolonged in response to lovastatin (Figure 5). The closure times increased an average of 2.45-fold (21.9 to 53.6 seconds) after incubation with lovastatin 200 nmol/L (P<0.05).

There were no significant prolongations of the ear bleeding time or ACT in response to lovastatin. There was no objective evidence of bleeding throughout the experiments. CBC indices obtained preoperatively, intraoperatively, and at the end of the preparation did not change with respect to red cell, white cell, and platelet indices.

**Discussion**

The principal finding of the present study is that intravenous statin therapy acutely alters platelet-rich thrombus formation.
After a standardized arterial injury in a porcine model. Within just 30 minutes of intravenous infusion, the administration of mevinolin (lovastatin) significantly reduced platelet deposition at the arterial injury site. To our knowledge, neither the rapidity of such a response nor the route of drug delivery has previously been reported in relationship to arterial thrombosis. This response appears to be at least partly secondary to statin-induced inhibition of platelet function. Previous investigators have demonstrated that statins inhibit collagen-induced platelet CD40L expression and release, inhibition of ADP induced P-selectin expression and platelet aggregation and that statins augment ADP induced platelet nitric oxide release further limiting the prothrombotic properties of platelets. Whereas thrombus generation in our injury model has previously been determined to be highly thrombin-dependent, we sought to assess the impact of mevinolin on thrombin-induced platelet dense granule release reaction in vitro. After 30 minutes of incubation, thrombin-induced platelet dense granule secretion was significantly reduced. Statins are known to alter platelet activity including inhibition of platelet protease-activated thrombin receptor-1 (PAR-1) expression and activity. Similar inhibition of dense granule release was noted after stimulation with the platelet agonist, convulxin. Convulxin, a nonenzymatic glycoprotein venom isolated from Crotalus durissus terrificus, stimulates platelets through the collagen receptor, glycoprotein VI (GPVI) binding site. Simvastatin produced similar results suggesting that this effect is not specific to lovastatin but rather a class effect of statins in general. The specific pathway responsible for these platelet inhibitory effects is not completely clear. The effect of statins may be localized upstream of these receptors within a central signaling pathway. There is a growing body of evidence that inhibition of HMG-CoA reductase alters the generation of geranyl-geranyl pyrophosphate and farnesyl pyrophosphate, prenylated protein intermediaries essential to cell signaling and vesicular transport. By inhibiting generation of these proteins, statins may limit signal transduction following stimulation. This hypothesis is further supported by the finding that the inhibitory effects of statins on platelets can be reversed by preincubation with geranylgeranylpyrophosphate. Coincubation of platelets with farnesyl pyrophosphate did not reverse the inhibitory impact of either statin, however the dense granule ATP secretion was augmented by 40% in control platelets incubated with farnesyl pyrophosphate alone. This finding suggests that farnesyl pyrophosphate is an important mediator of platelet functionality, supplementation can augment normal platelet function, and that it is rapidly absorbed into these cells.

Beyond platelets, statins may inhibit plasmatic pathways of thrombus formation. Statins have been shown to reduce circulating levels of von Willebrand factor and factor VII in patients with ischemic heart disease. When used for 3 months, simvastatin reduced prothrombin activation, factor Va generation, fibrinogen content, factor XIII activation, and augmented factor Va inactivation. After 1 month, atorvastatin 40 mg/d attenuated blood coagulation at the site of vascular injury in patients with angiographically confirmed coronary artery disease. Measures of prothrombin activation were reduced by more than 50% in these individuals. In endothelial cell culture experiments, pitavastatin increased thrombomodulin antigen expression and mRNA levels without inducing tissue factor mRNA. These results could be reversed by administration of mevalonate or by geranylgeranylpyrophosphate supplementation. Furthermore, statins have been shown to downregulate tissue factor expression in monocytes, endothelial cells, and atherosclerotic plaques. If this mechanism is contributing to the findings of our study, it would indicate that this downregulation occurs rather rapidly.

Statins may also impact fibrinolytic pathways. Simvastatin reduced the expression of PAI-1 in human smooth muscle and endothelial cell cultures within 16 hours of incubation. Incubation of simvastatin in vitro with these cell types reduced the amount of PAI-1 antigen released from smooth muscle cells and endothelial cells that were costimulated with platelet-derived growth factor or transforming growth factor-β. Moreover, simvastatin increased endogenous tPA release by more than 2-fold. The effects of simvastatin were ameliorated by coinubcation of geranylgeranylpyrophosphate, but not by farnesyl pyrophosphate, in the cell cultures, thus attributing the mechanism specifically to geranylgeranyl-modified intermediates.

Route of delivery, oral versus intravenous, greatly impacts the bioavailability of lovastatin. Therefore, dose comparisons of these 2 routes of delivery may not be valid. In our study, we targeted an in vitro and in vivo lovastatin concentration of 200 nmol/L after intravenous bolus and infusion. Although in vivo drug concentrations were not measured, the final plasma concentration was assumed to be approximately 100 ng/mL assuming an average plasma volume of 2 L per animal. With oral administration, lovastatin undergoes extensive first pass hepatic metabolism with only approximately 5% of the active compound remaining in the circulation thereafter. Postclinical comparison of 40 mg daily immediate and slow release lovastatin given orally in 76 patients with moderate hyperlipidemia was performed to determine the pharmacokinetic differences between these 2 preparations. Both lovastatin and lovastatin acid were measured and active compound was distinguished from total HMG-CoA reductase inhibitor concentration. The maximal concentration of active inhibitor

![Figure 5. Platelet reactivity test assay. Chamber closure times were significantly prolonged in response to Lovastatin 200 nmol/L.](http://atvb.ahajournals.org/download/1274/Arterioscler-Thromb-Vasc-Biol-SB-9298.png)
varied from 6 to 27 ng/mL. The area under the curve varied from 136 to 256 ng/mL. Whereas each pig in our study weighed 40 kg on average, the total dose per animal was 8 mg lovastatin. Yet without hepatic first pass metabolic clearance, the concentration of circulating active drug likely exceeded that which occurs with the clinical use of lovastatin given orally.

A second important observation of our study is that when 2 arteries are injured sequentially within 30 minutes, the volume of thrombus within the second injured artery is more than 50% greater than the thrombus volume within the first injured artery. Using a simple, reproducible and well-characterized porcine model of acute arterial injury, we have previously demonstrated the existence of a propensity for arterial thrombosis which varies nearly 20-fold between animals but is relatively constant within an animal.34 We have demonstrated that this wide interanimal variation with thrombosis to a standardized injury is dependent on whole blood and not blood vessel variables.35 When both carotid arteries are injured simultaneously, platelet deposition is remarkably similar in the paired carotid arteries.34–36 We now show that the basal “predisposition” to arterial thrombosis can be modified or amplified by acquired variables. Our combined data support the hypothesis that one arterial injury influences the platelet deposition at a later second arterial injury site. We have speculated that platelets, leukocytes, and other circulating humoral elements encountering a first arterial injury site are likely modulated in such a way as to enhance thrombogenesis locally and globally within the circulation. At carotid flow rates of 120 to 200 mL/min, 5% to 8% of the total blood volume passes through the injured artery each minute so there is ample opportunity for exposure of the entire blood volume to the injured arterial site. One might conclude that by performing arterial injuries in series, we have now provided a unique model of secondary prophylaxis for arterial thrombosis. Still to be clarified is the effect of the number and extent of serial arterial injuries on the magnitude of final thrombotic response. This phenomenon may explain the clinical observation that patients suffering acute coronary syndromes or strokes are more likely to sustain a subsequent thrombotic event shortly after their initial event.37,38

The relevance of this second observation may further elucidate the impact of statin therapy for patients suffering acute coronary syndromes (ACS). There are a number of studies now demonstrating a significant likelihood of multiple culprit plaques in patients with ACS. Additionally, nearly all of the secondary prevention studies of statin therapy demonstrate a benefit with regard to reduced coronary ischemia and ischemia driven end points. Our data may provide an explanation as to why patients with acute arterial thrombosis appear to benefit from more aggressive and higher doses of statins compared to those receiving primary prophylaxis. The higher doses may more aggressively alter thrombotic responses, in part, because of these pleiotropic effects.

The data in this report demonstrate for the first time that acute intravenous administration of mevinolin (lovastatin), a statin now generically available, is associated with favorable alterations in platelet function including im-

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Arterioscler Thromb Vasc Biol. published online August 10, 2009; Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2009/08/10/ATVBAHA.109.190884.citation

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