Atorvastatin Delays Murine Platelet Activation In Vivo Even in the Absence of Endothelial NO Synthase

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Objective—Statins decrease mortality in patients with vascular disorders, and evidence for the pleiotropic effects of statins is accumulating. Statins enhance endothelial NO synthase (eNOS) expression, thereby attenuating platelet activation and thrombus formation. Our goal was to determine whether statins have eNOS-independent effects on platelet activation.

Methods and Results—Wild-type and eNOS-deficient mice were given a 14-day course of oral atorvastatin, and platelet activation was evaluated in vitro and in vivo. Whereas in wild-type mice atorvastatin inhibited platelet activation in vitro in response to numerous agonists, in eNOS-deficient mice, atorvastatin inhibited only thrombin-induced and protease-activated receptor 4 agonist peptide–induced platelet activation. Consistent with an eNOS-independent effect, atorvastatin inhibited platelet activation in vivo in both wild-type and eNOS-deficient mice.

Conclusion—Atorvastatin inhibits platelet activation via eNOS-dependent and eNOS-independent mechanisms with the latter restricted to protease-activated receptor 4–induced activation downstream to the receptor.

Key Words: atorvastatin ■ endothelial NO synthase ■ intravital microscopy ■ platelet activation ■ prenylation ■ protease-activated receptor 4

Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, effectively reduce the burden of atherothrombotic disease. Recent evidence suggests that this may be partly attributed to processes that are independent of lipid reduction. Statins seem to attenuate platelet activation in hypercholesterolemic patients and in animal models. Normocholesterolemic mice that received statins were protected in stroke and myocardial infarction models; they also exhibited decreased endothelial Rho GTP-binding activity, increased endothelial and platelet endothelial NO synthase (eNOS) mRNA expression, and lower circulating levels of platelet activation markers, including β-thromboglobulin and platelet factor-4.

NO inhibits platelet activation through a variety of mechanisms (see review by Loscalzo). NO activates guanylate cyclase in platelets, leading to an increase in cyclic-GMP (cGMP). This can alter many signaling proteins in platelets and is considered to be the major mediator of NO signal transduction. Rosuvastatin treatment of rats reduced phosphorylation of platelet vasodilator-stimulated phosphoprotein, an indicator of NO activity. Platelets from rats treated with cerivastatin exhibited reduced platelet aggregation and greater NO release, and these rats had delayed times to occlusion in a carotid artery injury model of thrombosis—all of these effects are abolished with L-arginine methyl ester, an NO inhibitor. In vitro, simvastatin inhibits collagen- and arachidonic acid–induced aggregation of rabbit platelets and enhances NO and cGMP production—effects that are attenuated with NO scavengers or inhibitors. Together, these findings provide evidence that statins inhibit platelets in an NO-dependent fashion. However, it remains unclear whether statins also have NO-independent effects on platelets. To explore this possibility, we compared the effects of a 14-day course of oral atorvastatin on in vitro and in vivo platelet function in eNOS-deficient mice with those in wild-type mice.

Methods

Mice

Wild-type C57BL/6J and eNOS-deficient (NOS3tm1Unc, stock 002684) male mice were obtained from Jackson Laboratories. All mice were at least 8 weeks old and weighed at least 20 g. The experimental group received a diet prepared by Dyets Inc (Bethlehem, PA) containing 0.03 g atorvastatin per kilogram of chow. To prepare the chow, atorvastatin tablets were ground into a powder, which was then mixed with the chow ingredients (AIN-76A) before compression into pellets. Control mice received the same mouse chow, but without the active drug. All mice consumed ≈5 g of chow per day, such that the experimental group received around 7.5 mg/kg of atorvastatin per day. Studies were done according to Canadian Council of Animal Care Guidelines, and all animal use protocols were approved by the Animal Research Ethics Board at McMaster University.

Isolation of Washed Mouse Platelets

Blood (1 mL) obtained by carotid cannulation was collected into 0.1 mL of 20 mmol/L Tris-HCl, pH 7.3, 137 mmol/L NaCl (tris-buffered saline) containing 20 U/mL heparin (Leo Pharma Inc, Sweden) and centrifuged at 80 g for 2 min. Washed platelets were then resuspended in normal saline and used immediately for experiments.
Thornhill, ON, Canada). Platelet-rich plasma was obtained by centrifugation at 500g for 5 minutes. After a second centrifugation at 1500g for 5 minutes, the platelet pellet was washed twice in Tyrode buffer containing 20 mM/L apyrase (Sigma) and 0.5 mmol/L prostacyclin (Calbiochem, San Diego, CA) and then resuspended in 500 µL of the same buffer supplemented with 40 mM/L apyrase. Experiments with washed platelets were performed after they were incubated for 30 minutes at 37°C; this method is described in detail in the Emfret Analytics Web site (www.emfret.com).

Determination of Platelet cGMP Levels
Platelets were obtained and washed as described above. After lysis of 1x10^7 washed platelets with 0.1 mol/L HCl, the cellular debris was pelleted by centrifugation at 600g. After acetylation using acetic anhydride and triethylamine, cGMP in the supernatant was assayed using a competitive ELISA (Stressgen). To reduce the potential of between-day variation, samples were analyzed in pairs (wild-type and eNOS-deficient or control and atorvastatin-treated), and results were expressed relative to the values for wild-type mice or on a control diet.

Flow Cytometry Assessment of Platelet Activation
Activation of washed mouse platelets with ADP (0.12 to 121 µmol/L final concentration, Sigma), with thrombin (2.4 to 12.1 µmol/L final concentration, Roche Diagnostics), with H-Gly-Tyr-Pro-Gly-Lys-Phe-OH (GYP), a protease-activated receptor 4 (PAR4) agonist peptide (1.67 to 5.28 mmol/L final concentration, Bachem), or with collagen (33.3 to 216 µg/mL final concentration, Nycomed Pharma, Ismaning, Germany) was monitored by flow cytometry, using JONA-PE (an antibody that recognizes only the activated form of αIIbβ3, Emfret Analytics) and anti-P-selectin-ITC (Wigm, Emfret Analytics) at recommended concentrations. Values were expressed as mean fluorescence intensity.

Multiplate Determination of Platelet Activation
Blood samples were taken via carotid cannulation into 3.2% sodium citrate (9.1 vol/vol), and 175-µL aliquots were mixed with an equal volume of normal saline containing 1.5 mmol/L CaCl2 in pediatric cuvettes. After 3-minute incubation at 37°C, aggregation was initiated by an addition of 162 U/L thrombin (Sigma) or 7 µg/mL collagen (Nycomed Pharma), and impedance was continuously monitored for 6 minutes using a Multiplate Analyzer (Diapharma). Values were expressed as area under the curve. In some experiments, 4 mmol/L aspirin was incubated with the blood for 10 minutes at room temperature before recalcification.

PAR4 Expression on Mouse Platelets
PAR4 expression on washed mouse platelets was quantified by flow cytometry using 53 µg/mL of sc-8461, a polyclonal goat antibody directed against the N terminus of mouse PAR4 (Santa Cruz), or irrelevant goat IgG, and detected with 53 µg/mL of PE-conjugated anti-goat IgG, a secondary antibody (Santa Cruz) directed against the primary antibody. X488 (Emfret Analytics), a monoclonal antibody directed against GPIBα, and tagged with DyLight-488, was used to gate the platelet population. Values were expressed as geometric mean fluorescence intensity.

Intravital Analysis of Platelet Accumulation and Activation After Laser-Induced Injury to Cremaster Muscle Arterioles
In anesthetized mice, the cremaster muscle was prepared as previously described.24 Using the jugular vein cannulus, mice were given an infusion of 0.1 µg/g X488. Where indicated, an infusion of 1.3 µg/g of DyLight-647-tagged F(ab)2 fragments of rat anti-mouse CD41 (MWREG30, Emfret Analytics), a monoclonal antibody directed against αIIb that recognizes both the resting and activated form of this platelet-specific integrin, or 2 µg/g of DyLight-647-tagged antibody against P-selectin (RB40.34, Becton Dickinson) was also administered.25 Fluorophore conjugation and F(ab)2 fragment preparation were performed according to the directions from the supplier (Pierce). After laser injury to cremaster arterioles, platelet accumulation and activation were measured and analyzed as previously described,24 except that platelet activation was assessed on the basis of both CD41 upregulation and P-selectin surface expression. Three-dimensional movies were generated using Slidebook (Version 5, Intelligent Imaging Innovations, Denver, CO).

Statistical Analyses
Data were analyzed using Prism 5.0. Agonist-induced platelet activation detected by flow cytometry (using JONA-PE and anti–P-selectin) was fitted to a sigmoidal dose–response curve with 4 parameters and a fixing bottom at 0. The significance of difference between fitted curves was examined using an extra sum of squares F test (Prism). Group data were expressed as means±SEM. Significance of differences was determined using Student t test. cGMP levels were normalized relative to control values, and significance of the means relative to 1.0 was determined using t tests. In all analyses, P<0.05 was considered statistically significant.

Results
Atorvastatin Treatment Increases Platelet cGMP Levels
cGMP levels were measured using a competitive ELISA. Compared with controls, atorvastatin treatment increased platelet cGMP levels by 1.5-fold (95% CI, 1.3- to 1.7-fold) in wild-type mice. In contrast, cGMP levels in eNOS-deficient mice were 33.6% lower than those in wild-type mice (95% CI, 18.5%–48.6%) and were not changed with atorvastatin treatment (95% CI, 0.86- to 1.28-fold). Thus, atorvastatin increases cGMP in platelets from wild-type mice, but not in platelets from eNOS-deficient mice, consistent with the known capacity of statins to upregulate eNOS activity.

Atorvastatin Treatment Inhibits Platelet Activation In Vitro Even in Platelets From eNOS-Deficient Mice
In platelets from wild-type mice, atorvastatin treatment inhibited platelet activation induced by ADP, collagen, GYP, and thrombin as measured by αIIbβ3 activation (JONA-PE binding), and by thrombin and GYP as measured by anti–P-selectin binding (Figure 1). ADP did not increase anti–P-selectin binding (data not shown), and collagen-induced P-selectin expression was not inhibited by atorvastatin. In eNOS-deficient mice, atorvastatin treatment significantly inhibited platelet activation by collagen, GYP, and thrombin as measured by JONA-PE binding, and by thrombin and GYP as measured by anti–P-selectin binding (Figure 2). In these mice, atorvastatin did not significantly alter platelet activation by ADP as measured by JONA-PE binding or by collagen as measured by anti–P-selectin binding (Figure 2). Thus, although atorvastatin inhibits platelet activation in response to a wide range of agonists in wild-type mice, it only inhibits platelet activation in response to thrombin or GYP in eNOS-deficient mice. Likewise, in the Multiplate Analyzer, atorvastatin significantly reduced (P=0.04) the area under the curve of aggregation induced by thrombin in whole blood from eNOS-deficient mice, but had no significant effect on collagen-induced aggregation (Figure 3A). To interrogate the
Figure 1. Effect of atorvastatin diet on the flow cytometry markers of platelet activation in wild-type mice. Washed platelets were obtained from mice fed with atorvastatin diet 0.03 g/kg (triangles, dashed lines) or control chow (squares, solid lines) for 14 days. Data show means±SEM, n=14 mice per group. The x axis depicts the log of the agonist concentration. Best-fit sigmoidal dose–response curves were generated using Prism, assuming a bottom of 0. P values are an extra sum of squares F test of the null hypothesis that the 3 parameters (slope, EC50, and top) are same between the groups. If the 3 parameters are the same (P>0.05), then the shared fit is drawn as a dash-dot line. A, JONA-PE binding after activation by ADP, GYP, collagen, or thrombin. B, Anti–P-selectin binding in the same samples. MFI indicates mean fluorescence intensity.

effects of atorvastatin on the thromboxane pathway, platelets from eNOS-deficient mice were treated with aspirin in vitro before Multiplate analysis. Aspirin pretreatment decreased the area under the curve induced by collagen in atorvastatin-treated mice to the same extent as it did in control mice (Figure 3B). Taken together, these results suggest that in eNOS-deficient mice, atorvastatin treatment inhibits thrombin- and GYP-induced activation. In contrast, in these mice, atorvastatin

Figure 2. Effect of atorvastatin diet on the flow cytometry markers of platelet activation in endothelial NO synthase (eNOS)-deficient mice. Washed platelets were obtained from eNOS-deficient mice fed with atorvastatin diet (triangles, dashed lines) or control chow (squares, solid lines) for 14 days. Data show means±SEM, n=14 mice per group. The x axis depicts the log of the agonist concentration. Best-fit sigmoidal dose–response curves were generated using Prism assuming a bottom of 0. P values are an extra sum of squares F test of the null hypothesis that the 3 parameters (slope, EC50, and top) are same between the groups. If the 3 parameters are the same (P>0.05), then the shared fit is drawn as a dash-dot line. A, JONA-PE binding after activation by ADP, GYP, collagen, or thrombin. B, Anti–P-selectin binding in the same samples. MFI indicates mean fluorescence intensity.
does not influence collagen-induced activation nor does it seem to alter the thromboxane pathway.

**PAR4 Expression on Platelets Is Not Altered by Atorvastatin Treatment**

To exclude the possibility that the impaired response to GYP and thrombin in platelets from atorvastatin-treated mice reflects reduced expression of PAR4 on their platelets, PAR4 expression on platelets from wild-type mice was quantified using flow cytometry with a PAR4-directed antibody. Although the fluorescence intensity of PAR4 antibody binding to the resting platelets was low, it was above background and was similar in atorvastatin-treated mice and in the untreated controls (n=10 per group; Figure 4).

**Atorvastatin Treatment Has No Effect on Platelet Accumulation in Thrombi In Vivo**

Platelet accumulation in laser-injured cremaster arterioles was assessed using established methods. In both wild-type and eNOS-deficient mice, atorvastatin had no effect on the integrated intensity of GPIbβ, maximal intensity, or time to maximal intensity (data not shown).

**Time to Half-Maximal Activation Ratio of CD41 and P-Selectin of Platelets in Thrombi Is Delayed by Atorvastatin Treatment of Wild-Type Mice**

Because atorvastatin had no effect on platelet accumulation after laser-induced injury in wild-type mice, we examined the effect of atorvastatin on platelet activation in thrombi formed in wild-type mice (Figure 5). Compared with controls, atorvastatin treatment significantly (P<0.0001) prolonged the time to half-maximal activation ratio of CD41 1.5-fold from 98±6 seconds (n=33) to 150±9 seconds (n=46). The time to half-maximal activation ratio of P-selectin (Figure 5) was also significantly prolonged (P=0.0044) by atorvastatin treatment by 1.2-fold from 132±5 seconds (n=62) to 158±7 seconds (n=44). Thus, although atorvastatin does not alter platelet accumulation in the thrombi that form at sites of laser injury, it delays platelet activation events within these thrombi.

**Discussion**

There is mounting evidence that statins inhibit platelet activation in an eNOS-dependent fashion. This is not surprising given that NO is a potent inhibitor of platelet activation. However, it is unknown whether statins have eNOS-independent effects on platelets. To address this possibility, we compared the effects of atorvastatin on platelet activation in vitro and in vivo in mice deficient in eNOS.
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wild-type mice. Atorvastatin treatment of eNOS-deficient mice inhibited platelet activation induced by thrombin and by GYP, inhibited aggregation induced by thrombin, and delayed platelet activation in thrombi, as measured by CD41 upregulation.

This report adds to the literature supporting the inhibition of platelet activation by statins, specifically a chronic oral dose of atorvastatin. The mice consumed ≈7.5 mg/kg body weight of atorvastatin per day, but because of poor oral absorption of atorvastatin in mice, reported plasma levels in mice are lower than what would be expected with the same dose in humans.10,22,26,27 A higher dose of atorvastatin inhibited ADP- and PAR-induced platelet activation in hyperlipidemic patients with coronary artery disease, whereas a lower dose of atorvastatin plus ezetimibe did not, even though both treatment regimens reduced low-density lipoprotein cholesterol to a similar extent,28 implying that statin effects on platelets might be dose dependent and not related to lipoprotein level. Recent studies have also shown that intravenously administered statins have acute effects on platelet function in vitro and in vivo.29,30

The effect of statins on eNOS is well studied. NO, synthesized from L-arginine by NO synthase, activates intracellular soluble guanylyl cyclase and results in the formation of cGMP, an important modulator in many physiological and pathological conditions.31 It is important that in platelets, the NO/cGMP system has been shown to provide an inhibitory pathway-regulating platelet adhesion and aggregation.14-17,19,20,32 Also, statins increase NO bioavailability in vascular cells and platelets by enhancing eNOS expression, stabilizing eNOS mRNA, and possibly decreasing superoxide formation,13,33-35 suggesting that induced NO formation may be the major mechanism of the platelet inhibitory activity of statins. At least 2 studies have sought an eNOS-independent vascular protective effect of statins. The same dosing regimen of atorvastatin that we used lowered the plasma levels of platelet factor-4 and β-thromboglobulin (markers of platelet α-granule release) in wild-type mice but had no effect on these parameters in eNOS-deficient mice.36 Thus, our demonstration that atorvastatin inhibits platelet activation in eNOS-deficient mice is important, because we evaluated platelet function directly to reveal effects that were not seen with indirect measurements of platelets activation.

Because the comparison of the effects of atorvastatin treatment on platelet activation in vitro was evaluated in pairs, we are unable to make firm conclusions about the effect of eNOS-deficiency per se (compared with wild-type mice) on these parameters of platelet activation. The Loscalzo group has reported that platelets from eNOS-deficient mice are hyper-reactive.16 Consistent with this concept, we show that cGMP levels were lower in eNOS-deficient mice than in wild-type mice. Also, platelet activation in vivo, as measured by CD41 upregulation, occurred more rapidly in eNOS-deficient mice that were given a control diet than in their wild-type mouse counterparts. Using the same comparison, atorvastatin prolonged the time of half-maximal CD41 upregulation by 1.5-fold in wild-type mice and 1.3-fold in eNOS-deficient mice, implying that around half of the atorvastatin effect on this parameter is eNOS independent.
That atorvastatin inhibits platelet activation in the absence of eNOS broadens the clinical importance of statins as platelet inhibitors. In patients with cardiovascular risk factors, eNOS can be uncoupled. Uncoupling refers to the state where eNOS products combine with products of nicotinamide adenine dinucleotide phosphate-oxidases to form peroxynitrite, which oxidizes (6R)-5,6,7,8-tetrahydro-L-bioterin, an essential eNOS cofactor. In the absence of 5,6,7,8-tetrahydro-L-bioterin, eNOS produces superoxide instead of NO, which contributes to vascular stress. Our findings predict that the antiplatelet effect of statins might persist in patients with metabolic syndrome even though eNOS is uncoupled and NO production is impaired.

Our observation that atorvastatin inhibits thrombin- and GYP-induced activation in platelets from eNOS-deficient mice is novel. The effects of statins on PAR-signaling have been previously reported in humans. In patients with metabolic syndrome, statins have been reported to reduce PAR1 (the dominant PAR on human platelets). In our study, statin treatment had no effect on PAR4 expression. Although this may reflect differences in the effect of statin treatment on human and mouse platelets, the inhibited platelet activation that we observed in eNOS-deficient mice seems to reflect events downstream to the PAR4 receptor because thrombin- and GYP-induced platelet activation was inhibited. This concept is supported by studies with human platelets that demonstrate inhibition of PAR1 activation after 2 hours of incubation with a statin. In these studies, addition of prenyl substrates restored PAR1 activation, suggesting that impaired prenylation downstream to PAR1 may be responsible for the statin effect. Alternatively, the eNOS-independent effects of statins might involve PPARγ because simvastatin has been reported to inhibit platelet protein kinase C or via interactions with PPARγ. In this report, the PPARγ-dependent effect of simvastatin was observed when platelets were activated by arachidonic acid or ADP. In our studies, atorvastatin inhibited only ADP-induced activation in wild-type mice, raising the possibility that if this pathway depends on PPARγ, it may also require the presence of eNOS.

Although atorvastatin did not inhibit platelet accumulation, it inhibited the upregulation of markers of platelet activation in thrombi formed in both eNOS-deficient and wild-type mice. This finding provides further validation for this high-speed, 2-color microscopy method for evaluating the effects of inhibitors on the markers of platelet activation in vivo. The observation that atorvastatin delayed CD41 upregulation in eNOS-deficient mice, despite the fact that it only inhibited thrombin- and GYP-induced platelet activation, is consistent with previous observations that platelet activation in the laser-induced arteriolar model is thrombin dependent.

In conclusion, we have extended our understanding of how statins inhibit platelet activation. Independent of the effects on eNOS, atorvastatin inhibits thrombin- and GYP-induced platelet activation without altering the expression of PAR4.

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Disclosures
None.

References
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