HMG-CoA reductase inhibitor attenuates platelet adhesion in intestinal venules of hypercholesterolemic mice

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Tailor, Anitaben, David J. Lefer, and D. Neil Granger. HMG-CoA reductase inhibitor attenuates platelet adhesion in intestinal venules of hypercholesterolemic mice. Am J Physiol Heart Circ Physiol 286: H1402–H1407, 2004. First published December 23, 2003; 10.1152/ajpheart.00993.2003.—Whereas the anti-inflammatory properties of statins have been extensively studied, less attention has been devoted to the antithrombogenic effects of these drugs. We evaluated the effect of short-term (18 h) treatment with pravastatin (1 mg/kg) on hypercholesterolemia-induced platelet-endothelial (P/E) cell adhesion in intestinal venules. Mice were placed on either a normal diet (ND) or cholesterol-enriched diet (HCD) for 2 wk. Wild-type mice fed a HCD exhibited significantly elevated blood serum cholesterol levels, which were unaltered by pravastatin treatment. ND or HCD platelets were isolated, fluorescently labeled, and administered to either ND or HCD recipients. Intravital videomicroscopy was used to quantify transient (saltation) and firm adhesion of platelets. HCD mice receiving platelets from either ND or HCD mice exhibited increased P/E cell interactions compared with ND mice receiving platelets from ND or HCD mice. P/E adhesion was dramatically reduced when platelets from donor mice, recipient mice, or both were treated with pravastatin. The protective effect of pravastatin in hypercholesterolemia-induced P/E cell adhesion was abolished in Nω-nitro-L-arginine methyl ester-treated mice. These results indicate that 1) hypercholesterolemia-induced P/E cell adhesion is mediated by changes in the vascular wall rather than circulating platelets; 2) pravastatin treatment inhibits the prothrombogenic effects of hypercholesterolemia via an action on both endothelial cells and platelets; and 3) the protective effect of pravastatin is nitric oxide dependent.

3-HYDROXY-3-METHYLGLUTARYL COENZYME A (HMG-CoA) reductase inhibitors (i.e., simvastatin, lovastatin, pravastatin, etc.) are widely used in clinical practice to lower serum cholesterol and to effectively reduce the cardiovascular related morbidity and mortality that is associated with hypercholesterolemia. Statins exert their cholesterol-lowering action by blocking the conversion of HMG-CoA to mevalonate, the substrate for 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase). Statins exhibit potent anti-inflammatory properties in the setting of ischemia-reperfusion (I/R) (11, 15, 41), hypercholesterolemia (19, 33), and diabetes (20, 27).

Hypercholesterolemia is a major risk factor for cardiovascular diseases that predisposes the vasculature to thrombogenesis and the development of atherosclerosis. The endothelial cell activation, dysfunction, and injury that can accompany hypercholesterolemia appear to favor the adhesion and arrest of circulating cells (leukocytes and platelets) onto the walls of large and microscopic blood vessels (4, 15, 22, 32). Several studies have shown that circulating platelets become hyperactive in the setting of hypercholesterolemia (5, 18, 26), and we (38) have recently demonstrated enhanced adhesion of platelets in unstimulated postcapillary venules of hypercholesterolemic mice. The adhesion of these activated platelets on the vessel wall can exacerbate the inflammatory phenotype of endothelial cells that is induced by hypercholesterolemia and may thereby contribute to the development and progression of cardiovascular diseases, such as atherosclerosis, thrombosis, and acute coronary syndromes (7, 22, 35). Although the pathophysiological consequences of platelet activation during hypercholesterolemia remain poorly understood, it is well established that platelet activation is associated with an enhanced risk for cardiovascular diseases.

Endothelial nitric oxide (NO) synthase (eNOS) is present in both endothelial cells and platelets, and its product NO can inhibit several components of the inflammatory process (8, 13, 29). One of the earliest manifestations of hypercholesterolemia is an impairment of NO production by endothelial cells (8, 16). The results of several recent studies indicate that statins improve endothelial cell function and inhibit platelet activation (10, 12, 34), presumably by increasing eNOS expression in endothelial cells and platelets, respectively (17, 24, 39). The results of a recent in vivo study (2) indicate that atorvastatin treatment attenuates the adherence of platelets to the eroded vessel walls of hypercholesterolemic swine, thereby reducing the thrombotic risk associated with atherosclerosis.

Although the ability of statins to inhibit leukocyte-endothelial cell adhesion has been demonstrated in postcapillary venules of hypercholesterolemic (15) and normocholesterolemic (28) animals, there are no published reports that describe an effect of statins on platelet-endothelial cell interactions under real-time conditions in any segment of the hypercholesterolemic vascular tree. Hence, a major objective of this study was to determine whether pravastatin, a widely used statin with documented anti-inflammatory properties (25, 31), alters the adhesion of platelets in the microvasculature of hypercholesterolemic mice and whether any antiadhesion effect of the statins reflects an action on circulating platelets, the vascular wall, or both. In addition, we addressed whether the effects of pravastatin on platelet-endothelial cell adhesion are mediated via an NO-dependent pathway.

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MATERIALS AND METHODS

Animals. Wild-type (WT) C57BL/6j mice (6–8 wk of age) were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were housed in a climate-controlled room, exposed to a 12:12-h light-dark cycle, and allowed free access to normal rodent chow. The experimental procedures used in this study were reviewed and approved by the Louisiana State University Health Sciences Center-Shreveport Institutional Animal Care and Use Committee and performed according to criteria outlined in National Institutes of Health guidelines.

Treatment. WT mice (n = 9) were placed on either a normal diet (ND) or cholesterol-enriched diet (HCD; Teklad 90221 containing 1.25% cholesterol, 15.8% fat, and 0.125% choline chloride; Harlan Teklad) for 2 wk. Animals were treated intraperitoneally with either vehicle (PBS, pH 7.4) or Pravastatin (1 mg/kg) 18 h before experiment and in all experiments. The preparation was allowed to stabilize for 10 min before infusion.

Platelet isolation. The carotid artery was cannulated, and blood was collected into acid-citrate-dextrose (38 mM citric acid, 75 mM trisodium citrate, and 100 mM dextrose, 1/10 vol). Platelet-rich plasma (PRP) was obtained by two sequential centrifugations (120 g for 8 min and 120 g for 3 min). PRP was removed and spun again at 550 g for 10 min, and the pellet was resuspended in PBS. Platelets were labeled with the fluorochrome carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes; Eugene, OR, final concentration: 90 μM), as previously described (38). The CFSE-labeled platelets were spun at 550 g, and the pellet was resuspended in PBS and stored at room temperature until use. To determine whether the isolation procedure altered platelet activation status, P-selectin expression was measured by flow cytometry. The separation procedure did not significantly alter P-selectin expression on washed platelets compared with platelets in fresh whole blood, as previously described (30, 38).

Surgical procedures and intravital fluorescence microscopy. Mice were anesthetized with a mixture of ketamine hydrochloride (150 mg/kg ip) and xylazine (7.5 mg/kg ip). The right carotid artery was cannulated for blood pressure measurement using a disposable pressure transducer (Cobe Laboratories) attached to a MacLab/4e and Quad Bridge. This equipment was attached to a MacLab (version 3.5.2, AD Instruments) blood pressure chart. The right jugular vein was cannulated for CFSE-labeled platelet infusion. A midline laparotomy was performed, and the animal was placed in the supine position. A loop of the small bowel was exteriorized and superfused with warm bicarbonate buffer solution (BBS; 132 mM NaCl, 4.69 mM KCl, 1.98 mM MgSO4, 20 mM NaHCO3, and 1.98 mM CaCl2). The preparation was allowed to stabilize for 10 min before infusion with CFSE-labeled platelets. Labeled platelets were infused (100 × 106) over a period of 5 min using a Harvard Apparatus (South Natick, MA) infusion pump, yielding ~5% of the total platelet count, and allowed to circulate for 5 min before the observation period. Platelets were visualized with an upright microscope (Nikon Diaphot 300) equipped with a 100-W mercury lamp with a ×20 objective lens (200/4; Fluor, Nikon). Microscopic images were received by a charge-coupled device videocamera (C2400, Hamamatsu) and were projected onto a color monitor (PVM-134MD, Sony Trinitron). The images were recorded on a VHS video recorder (AG-6730, Panasonic) for off-line analysis.

Image analysis. Three to five single unbranched postcapillary venules with a diameter of 25–40 μm and a minimal length of 200 μm were selected for study, and each vessel was recorded for 1 min. Platelets were classified according to their interaction with the venular wall as either saltating or firmly adherent platelets. Saltation was defined as platelets interacting with the endothelium for <30 s (2–29 s). Firmly adherent platelets were defined as platelets stationary on the venular wall for >30 s. Adhesion data are expressed as the number of platelets per square millimeter of endothelial surface, calculated from diameter and length, assuming cylindrical vessel shape (23).

To estimate wall shear rate in postcapillary venules, we substituted the maximal velocity of flowing platelets (Vplt) for the centerline red blood cell velocity. The mean red blood cell velocity was estimated as Vpurple. The venular wall pseudoshear rate was calculated based on Poiseuille’s law for a Newtonian fluid: pseudoshear rate = (Vpurple/D) × 8, where D is the venular diameter (3).

Serum cholesterol levels. Serum samples collected from each mouse were frozen (~80°C) for subsequent measurement of total cholesterol levels using a spectrophotometric assay (Sigma).

Statistical analysis. Data were analyzed by standard statistical analysis, i.e., one-way ANOVA with Scheffe’s post hoc test. All values are reported as means ± SE, from 5–9 mice/group, and statistical significance was set at P < 0.05.

RESULTS

Effect of pravastatin on blood cholesterol levels. Mice placed on a cholesterol-enriched diet for 2 wk exhibited a significant increase in total cholesterol levels compared with their ND counterparts (172 ± 76.4 vs. 73 ± 7 mg/dl). Treatment with pravastatin did not alter total cholesterol levels in any of the groups studied (Table 1).

Effects of hypercholesterolemia on platelet-endothelium interactions. Figure 1 summarizes the results of experiments designed to address the relative contribution of the vascular wall and circulating platelets to the platelet-endothelial cell (P/E) adhesion observed in intestinal venules of mice placed on a cholesterol-enriched diet (2 wk). When platelets harvested from mice placed on a ND were monitored in HCD recipients, no platelet adhesion was seen in arterioles, whereas a profound (9-fold) increase in the total number of adherent platelets (saltating and firmly adherent) was noted in venules compared with the response seen when ND recipients received ND platelets. Similarly, when HCD platelets were monitored in HCD recipients, platelet adhesion was significantly increased 11-fold. In contrast, when HCD platelets were observed in ND recipients, a slight increase in P/E cell adhesion was noted, but this response was not statistically different from ND mice receiving ND platelets (Fig. 1).

Because it has recently been shown that venular shear rate can influence the magnitude of P/E cell adhesion in postcapillary venules (30), we estimated the venular pseudoshear rate in all experiments. The results revealed no statistically significant difference in pseudoshear rates between any of the

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Pseudoshear Rate, s⁻¹</th>
<th>Total Serum Cholesterol, mg/dl</th>
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</thead>
<tbody>
<tr>
<td>ND → ND</td>
<td>711 ± 21</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>HCD → HCD</td>
<td>600 ± 35</td>
<td>172 ± 15*</td>
</tr>
<tr>
<td>HCD → HCD + statin</td>
<td>565 ± 27</td>
<td>168 ± 9*</td>
</tr>
<tr>
<td>HCD + statin → HCD</td>
<td>692 ± 60</td>
<td>180 ± 15*</td>
</tr>
<tr>
<td>HCD + statin → HCD + statin</td>
<td>528 ± 38</td>
<td>156 ± 16*</td>
</tr>
<tr>
<td>HCD + l-NAME → HCD + l-NAME</td>
<td>745 ± 59</td>
<td>155 ± 13*</td>
</tr>
<tr>
<td>HCD → HCD + l-NAME + statin</td>
<td>690 ± 36</td>
<td>187 ± 12*</td>
</tr>
</tbody>
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Values are means ± SE; n = 5–8 animals/group. ND, normal diet; HCD, high-cholesterol diet; l-NAME, Nω-nitro-l-arginine methyl ester. *Statistical difference (P < 0.05).
experimental groups, with mean values ranging between 528 ± 38 and 745 ± 59 s⁻¹ (Table 1). Thus the adhesive interactions observed between platelets and endothelial cells were not attributable to hemodynamic factors.

**Effect of pravastatin on hypercholesterolemia-induced P/E cell adhesion.** Figures 2 and 3 summarize the findings from experiments designed to address the influence of pravastatin treatment on hypercholesterolemia-induced platelet salta-tion (Fig. 2) and firm adhesion (Fig. 3). When HCD platelets from untreated mice were monitored in HCD mice treated with pravastatin, highly significant reductions (60–90%) in the number of salting and firmly adherent platelets were noted compared with untreated HCD platelets in untreated HCD recipients. Similar attenuation of the hypercholesterolemia-induced P/E cell adhesion responses were noted when platelets from pravastatin-treated HCD mice were monitored in either untreated or pravastatin-treated HCD recipients. For example, when both donor and recipient HCD mice were treated with pravastatin, platelet salitation was reduced by 55% (Fig. 2), whereas firm adhesion was reduced to near basal levels (Fig. 3).

**Effect of L-NAME on hypercholesterolemia-induced P/E cell adhesion in pravastatin-treated mice.** L-NAME treatment was used to determine whether the inhibitory effect of pravastatin on hypercholesterolemia-induced P/E cell adhesion was NO dependent. Inhibition of NOS function was assessed by alterations in mean arterial blood pressure after an intravenous administration of acetylcholine (10⁻⁵ M). Untreated animals receiving acetylcholine demonstrated a transient decrease (40%) in mean arterial blood pressure. The agonist-induced decrease in mean arterial blood pressure was reduced (12%) in L-NAME-treated mice. L-NAME did not alter mean arterial blood pressure compared with the respective treatment group (values ranging between 57 and 65 mmHg).

Our findings indicate that when both donor and recipient mice on a HCD for 2 wk were treated with L-NAME, P/E cell adhesion was elevated to a level comparable with that seen in nontreated hypercholesterolemic mice. In the presence of the NOS inhibitor, pravastatin treatment of recipient mice was not associated with an attenuation of either platelet salitation (Fig. 4) or firm adhesion (Fig. 5).

**DISCUSSION**

Hypercholesterolemia is widely recognized to be a major risk factor for the development of atherosclerosis and thrombosis-related vascular diseases, such as stroke and myocardial infarction. An important consequence of elevations in blood cholesterol is the activation of both circulating blood cells (e.g., leukocytes and platelets) and endothelial cells that line the walls of large and microscopic blood vessels. These activated cells are typically in a state of oxidative stress and exhibit an increased surface expression of glycoproteins that mediate
the adhesion of circulating blood cells to the vessel wall. As a result of these responses, the vasculature assumes a proinflammatory and prothrombogenic phenotype that facilitates the processes of atherogenesis and thrombogenesis. Although the availability of therapeutic agents that can slow the development and/or progression of hypercholesterolemia-induced atherogenesis and thrombogenesis remain limited, there is growing evidence that the statins may possess such therapeutic properties that are independent of their cholesterol-lowering action. The results of the present study demonstrate the ability of a widely used statin, pravastatin, to inhibit hypercholesterolemia-induced adhesion of platelets within the microcirculation and provide novel insights into the mechanisms underlying this antithrombogenic action of pravastatin.

We have recently demonstrated, using intravital fluorescence microscopy, that a large number of adherent platelets accumulate in postcapillary venules of mice placed on a HCD for a period of 2–8 wk. Flow cytometry revealed an increased expression of P-selectin on the surface of circulating platelets in hypercholesterolemic (HCD) mice compared with their normocholesterolemic (ND) counterparts. Furthermore, P-selectin-deficient mice were used to demonstrate a role for both platelet- and endothelial cell-associated P-selectin in mediating the enhanced platelet adhesion induced by hypercholesterolemia. The present study extends these observations to demonstrate that the P/E cell adhesion induced by hypercholesterolemia is largely mediated by changes in the vascular wall rather than circulating platelets. This conclusion is based on our observation that P/E cell adhesion occurs only when platelets derived from mice on either an HCD or ND are administered and monitored in HCD, but not in ND, recipient mice. This suggests that, despite the known ability of cholesterol exposure to render platelets hyperactive and to increase their expression of P-selectin (21, 26), these changes alone are not sufficient to elicit platelet adhesion to blood vessels. It is conceivable that the expression of counterreceptors for P-selectin and other adhesion molecules on the vessel wall is a more rate-determining step in hypercholesterolemia-induced P/E cell adhesion. Alternatively, a hypercholesterolemic extracellular environment may contain substances that either directly or indirectly promote P/E cell adhesion or suppress the production of agents that normally act to inhibit platelet adhesion (e.g., NO). Irrespective of the mechanism, our findings are consistent with numerous reports of human and animal experimentation that demonstrate endothelial cell dysfunction as a key component of the vascular responses to hypercholesterolemia.

The results of clinical trials have revealed that HMG-CoA reductase inhibitors (statins) offer protection by lowering cholesterol levels. However, it has become increasingly evident that these inhibitors exert their action independent of their cholesterol-lowering abilities against the tissue injury and/or microvascular dysfunction associated with hypercholesterolemia and I/R. The beneficial effects of statins in these and other pathophysiological states have been attributed to improved blood flow regulation, inhibition of leukocyte-endothelial cell adhesion, attenuation of oxidative stress, and/or enhanced vascular remodeling. The results of the present study add another potential mechanism to the list of vascular protective effects exerted by statins, i.e., inhibition of P/E cell adhesion. This previously unreported action of a statin also appears to be independent of the drug’s cholesterol-lowering effect, inasmuch as serum cholesterol concentration was unchanged in pravastatin-treated mice.

There is substantial evidence in the literature that there is diminished NO production during hypercholesterolemia. Furthermore, several reports have described a beneficial effect of statin therapy on endothelial cell as well as platelet function. For example, studies have revealed reduced staining for endothelial P-selectin expression after treatment with simvastatin (28) and rosuvastatin (36), with the latter linked to increased NO production. Hypercholesterolemic patients treated with pravastatin exhibit an attenuated platelet aggregation and a reduced platelet expression of P-selectin (21). Similarly, simvastatin (40) and atorvastatin (17) have been shown to blunt the platelet activation responses to different stimuli. In addition, the ability of atorvastatin to inhibit platelet activation is asso-
ciated with an upregulation of type III NOS (eNOS) in both platelets and vascular endothelial cells, suggesting that the elevated levels of NO generated by these cells may function to blunt the activation and aggregation (homotypic or heterotypic) of platelets. Our study revealed that hypercholesterolemia-induced P/E cell adhesion is reduced regardless of which cell type (endothelial cells and/or platelets) is exposed to statin treatment. We assume that the beneficial effects of statin therapy are mediated by an NO-dependent mechanism. The observation that the increased P/E cell adhesion was unaffected by pravastatin in the presence of L-NAME suggests that the protective action of pravastatin is dependent on the release of NO and that NO is important in regulating hypercholesterolemia-induced P/E cell responses. It remains unclear, however, whether the beneficial actions of the statin on both target cells (platelets and endothelial cells) are entirely dependent on NO production/bioavailability.

Two other known actions of the statins may contribute to the attenuation hypercholesterolemia-induced P/E cell adhesion, i.e., improved blood flow regulation and inhibition of leukocyte-endothelial cell adhesion. Statins have been reported to improve blood flow in some vascular beds (e.g., the brain) but not in others (e.g., the mesentery). An increased blood flow and the accompanying increase in venular shear rate would serve to reduce P/E cell adhesion because it has recently been shown that reduced shear rates promote, whereas elevated shear rates attenuate, P/E cell adhesion in venules (30). However, our estimates of venular shear rate support the view that pravastatin does not alter mesenteric blood flow and suggests that enhanced shear rate is an unlikely explanation for the anti-P/E cell effects of this drug.

The statins have been shown to exert profound inhibitory effects on leukocyte-endothelial cell adhesion in a variety of experimental models of inflammation. Because hypercholesterolemia appears to induce the adhesion of both leukocytes (32, 37) and platelets (38) in postcapillary venules, and platelets are known to bind to leukocytes (6, 9, 14) under certain pathological conditions, it is possible that pravastatin prevents hypercholesterolemia-induced platelet accumulation in venules through an action on leukocyte-endothelial cell adhesion. Hence, the statin could exert its anti-P/E cell adhesion effect by reducing either the adhesion of leukocytes to endothelial cells, the adhesion of platelets to adherent leukocytes, or both.

The pathophysiological significance of platelet adhesion in venules during hypercholesterolemia remains unclear. However, the accumulation of platelets in the microcirculation may well exacerbate the inflammatory phenotype and accelerate the endothelial cell dysfunction that is induced by hypercholesterolemia, which would render the vascular bed more vulnerable to ischemic injury. The dual action of statins to blunt platelet as well as leukocyte recruitment in the hypercholesterolemic microvasculature may explain the growing body of evidence for the significant cholesterol-independent protection against cardiovascular diseases that is afforded by prophylactic treatment with this class of drugs.

REFERENCES


GRANTS

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