Platelet Deposition on Eroded Vessel Walls at a Stenotic Shear Rate Is Inhibited by Lipid-Lowering Treatment With Atorvastatin

José Alfon, Teresa Royo, Xavier Garcia-Moll, Lina Badimon

Abstract—Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase are widely used in the treatment of dyslipemias and have shown beneficial effects in the primary and secondary prevention of cardiovascular diseases. However, regression studies with lipid-lowering drugs have not shown significant lesion reduction associated with the improvement in clinical events. Therefore, our objective has been to study whether treatment with a lipid-lowering drug of this family, atorvastatin, could reduce platelet deposition on the damaged vessel wall at different shear stress conditions, simultaneously with retardation of the development of atherosclerotic lesions. Using cholesterol-fed swine as the model, we found that atorvastatin significantly diminished platelet deposition on the mildly damaged vessel wall at high shear rates (50%, \( P<0.01 \)), but it did not have any effect in preventing platelet deposition triggered by a severely injured vessel wall. Development of coronary lesions was also reduced by treatment. These findings suggest that atorvastatin may prevent platelet attachment to eroded vessels and hence, contribute to reducing the thrombotic risk associated with the erosions of the luminal surface and the platelet-dependent progression of atherosclerotic plaques.


Key Words: HMG-CoA \( \text{reductase inhibitors} \), atorvastatin, platelet deposition, vessel wall

Clinical results with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) strongly suggest that the beneficial effects obtained with drugs of this family in cardiovascular disease are also related to factors beyond systemic lipid lowering and reduction in lesion size. In fact, cardiovascular episode reduction rates have a faster occurrence than does plaque regression.\(^1\) Statins have shown a broad spectrum of activities in vitro, such as inhibition of cell proliferation of different cell lines,\(^2\) inhibition of esterified cholesterol accumulation in smooth muscle cells,\(^3\) and inhibition of in vitro platelet aggregation.\(^4\) Atorvastatin is a new HMG-CoA reductase inhibitor that has shown efficacy in the treatment of hypercholesterolemia\(^5\) and hypertriglyceridemia.\(^6\) We have previously shown that atorvastatin reduces platelet deposition ex vivo in a severely dyslipemic rabbit model under flowing-blood conditions.\(^7\) Therefore, the objective of our study has been to evaluate the preventive effect of this statin on mural platelet deposition triggered not only by mildly damaged vessels but also by severely damaged (ruptured) vessel walls in a moderately hypercholesterolemic swine model. We found that coronary lesion development was reduced by treatment; in addition, platelet deposition was significantly inhibited by atorvastatin when the vessel wall exposed areas of erosion but not of rupture. Therefore, statins may act by reducing thrombotic risk and the progression of plaques by a platelet/fibrin-dependent process.

Methods

Study Design

Eight pigs (body weight, 17 ± 4 kg) (Granjas Tarradelles, Barcelona, Spain) were fed a cholesterol-rich diet (2% cholesterol, 1% cholic acid, and 20% beef tallow) for 8 weeks. Half of the animals simultaneously received 3 mg \( \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \) atorvastatin (a gift from Parke-Davis [Cristina Díaz, Barcelona, Spain]). Pigs underwent blood sampling at baseline (before starting treatment), 4 weeks after initiation of the study, and at the end of the study for hematological and biochemical determinations. At the end of 8 weeks, a sample of blood was withdrawn, and platelets were isolated, radioactively labeled, and reinjected. On the following day platelets were placed in the perfusion chamber, and platelet deposition and thrombus formation, triggered by the vessel wall with either erosion or rupture, were evaluated under various shear conditions. Animals were killed (KCl \( 2 \text{ mol/L}, 20 \text{ mL}, \text{carotid artery}) and samples from the coronary arteries and aorta were collected to measure intimal thickening. Platelet aggregation and von Willebrand factor (vWF) in samples from platelets and plasma were also measured. All procedures performed in this study were in accordance with institutional guidelines and followed the American Physiological Society guidelines for animal research.

Plasma Biochemistry

Blood was withdrawn from the femoral or carotid artery and collected into trisodium citrate (3.8%, 1/10 volume), and then plasma was obtained by centrifugation (15 minutes, 1200 \( \times \)g). Total cholesterol was determined with an automatic analyzer (Kodak Ektachem DT system). Lipoproteins (HDL cholesterol, LDL cholesterol, and VLDL cholesterol) were fractionated by using validated methods of the Lipid Research Clinics Program\(^8\) and quantified spectrophotometrically.
metically (Kontron Instruments). One atorvastatin (A) -treated animal was eliminated owing to abnormally elevated aspartate aminotransferase and alanine aminotransferase values and was not included in further analysis. In 1 control (C) animal, expected lipid values were not induced by the diet, and data for this animal were also eliminated. Coagulation parameters (prothrombin time, fibrinogen, and activated partial thromboplastin time) were analyzed with a coagulometer (ST4, Diagnostica Stago).

Platelet Labeling

On the day before the perfusion chamber experiment, 43 mL of blood was withdrawn in 7 mL of ACD (0.04 mol/L citric acid, 0.09 mol/L sodium citrate, and 0.07 mol/L dextrose), and platelets were labeled with $^{111}$In-oxine as previously described$^9$ and reinjected in a volume of 4 mL of plasma. In C and A-treated animals, total injected activity (C, 386±80 versus A, 329±58 μCi), total platelet count injected (C, 8.8±2.5×10$^9$ versus A, 11.1±1.8×10$^9$), and labeling efficiency (C, 81.3±4.1% versus A, 84.5±5.2%) were similar.

Platelet Deposition Experiment

Extracorporeal Shunt

On the day after platelet labeling, the pigs were sedated with intramuscular ketamine (20 mg/kg, Imalgene; Rhone) and xylazine (1 mg/kg, Rompun; Bayer), followed by intravenous infusion of sodium thiobitarbital. The carotid artery and contralateral jugular vein were catheterized by cutdown, and an extracorporeal circuit (carotid artery to perfusion chamber to jugular vein) was established as previously described.$^{10}$ Animals were heparinized (Liquemin, Roche) with a 50 U/kg IV bolus followed by a continuous infusion (50 μ·kg$^{-1}$·h$^{-1}$). Heparinization was monitored by the activated partial thromboplastin time ratio over baseline (C, 1.44±0.10 versus A, 1.56±0.39); the prothrombin time ratio over baseline (0.99±0.09) and plasma fibrinogen (185±32 mg/dL) were also continuously monitored and found to be similar between the 2 groups. The catheterized carotid artery was connected to the input of the chamber and the output to a peristaltic pump (Master-Flex, model 7518-10). Blood was perfused for 5 minutes over the vessel wall, and then buffer was passed through the chamber for 30 seconds under identical flow conditions to wash away unattached cells. Several perfusions were performed per animal. Differences in platelet deposition between substrates perfused at the beginning and the end of the experiments were not observed, indicating that platelet deposition was not affected by perfused blood reentering the circulation.

Shear Rate Conditions

Perfusions were performed under 2 different local-flow conditions to achieve low and high shear rates (212 s$^{-1}$ and 1690 s$^{-1}$, respectively), values typical of patent and stenotic arteries, respectively.$^{10}$ The low shear rate was achieved by perfusing at a 10 mL/min flow rate in the perfusion chamber with a 2-mm diameter, and the high shear rate was achieved at the same flow rate in a 1-mm-diameter perfusion chamber.

Triggering Vessel Wall Substrates

Thrombosis was triggered by porcine arterial vessel wall from the thoracic aorta. Aortas were collected from normal untreated animals, transported in PBS buffer, cleaned of adventitia, cut in 3-cm segments, and frozen. On the day of the experiment, aortic rings were opened longitudinally and cut into 1×3-cm segments. Two types of lesions were exposed to flowing blood in the perfusion chamber: aortic subendothelium (mild injury and tunica media (severe injury). The mildly injured arterial wall served as a model of endothelial erosion or denudation and was obtained by a single freeze-thaw cycle.$^9$ The aortic tunica media was used as a model for severe arterial injury; plaque disruption was mimicked by mechanically peeling off the intima together with a thin portion of the subjacent media, thereby exposing deeper components of aortic wall to flowing blood.$^{9,11}$

Quantification of Platelet Deposition

At the end of the perfusions, the radioactivity in each perfused vessel segment was quantified in a gamma counter, and the value obtained was converted to the number of deposited platelets×10$^6$ per unit of surface area as previously described.$^{10}$

Immunohistochemistry of Thrombus Formation

After perfusion in the flow chamber, tissues were fixed in 4% paraformaldehyde solution, cryoprotected with 2.3 mol/L sucrose, and frozen over dry ice in OCT (Tissue-Tek OCT compound 4583, Miles Inc.). Serially cut 6-μm sections in the direction of blood flow were obtained by using a cryostat (Leica), mounted on gelatinized slides, and examined for immunohistochemistry. As primary antibodies, we used a rabbit anti-vWF (1/1000; F3520, Sigma Immunochemicals) for platelet detection and a mouse anti-fibrin antibody (1/200; a gift from Dr Gaffney, National Institute of Biological Standards and Control (NIBSC), City, UK). Platelets can be specifically detected with vWF antibody because porcine endothelium does not releases vWF.$^{12}$ As secondary antibodies, we used an FITC-conjugated F(ab')$^2$ fragment of goat anti-mouse immunoglobulins (1/50; Dako, F479) and TRITC-conjugated swine anti-rabbit immunoglobulins (1/50; Dako, R156).

Lesion Development in Arteries

Segments from the right coronary artery, thoracic aorta (over the first intercostal branch), and abdominal aorta (at 2 levels: below the renal artery branch, higher abdominal; and over the iliac branch, lower abdominal) of pigs were readily dissected and excised just after euthanasia. The arteries were fixed, cryoprotected, OCT embedded, and cut as described above. Tissues were stained with Masson’s trichrome method$^{13}$ to identify and quantify vascular structures by morphometric analysis. The following parameters were measured with an image analyzer (Visilog): lumen area (L), area surrounded by the internal elastic lamina (IEL), and area surrounded by the external elastic lamina (EEL). Then the following parameters were determined: (1) intimal area=1−IEL−L; (2) medial area=I−EEL−IEL; (3) intima to media ratio=I/M; and (4) % stenosis=I/(L−I)×100.

Plasma and Platelet vWF

Platelets and plasma were collected when the animals were euthanized. Platelet (soluble and membrane) fractions were prepared by homogenizing washed platelets in a buffer with protease inhibitors and further centrifugation, as described elsewhere.$^{14}$ vWF was quantified in plasma and platelet fractions by ELISA by using the vWF standard from the NIBSC.$^{15}$ In brief, a 96-well plate was covered with anti-vWF (1/500; A0082, Dako) and reacted with plasma (1/20), the soluble platelet fraction (1/20), the platelet membrane fraction (1/20), a blank, or the standard (NIBSC, 0.96 IU/mL at several dilutions). Complexes were washed with PBS and reacted with 1/10,000 anti-vWF antibody coupled to horseradish peroxidase (P0226, Dako). The colorimetric substrate was o-diaminophenol dihydrochloride (P9187, Sigma). Absorbance was read at 492 nm.

Western Blot Analysis of Propolypeptide (pp)-vWF and vWF

A slab mini-cell (Bio-Rad) and the buffer system of Laemmli$^{16}$ were used to perform 7.5 SDS–polyacrylamide gel electrophoresis. The protein concentration was determined by the bicinchoninic acid protein assay (Pierce) with samples (20 μg) were heated to 100°C in a sample buffer that contained 2% SDS and 5% β-mercaptoethanol (reduced). Blotting was performed according to the method of Towbin et al$^{17}$ by using a Trans-Blot cell and nitrocellulose membranes (Bio-Rad). After transfer was completed, excess binding sites on the nitrocellulose sheets were blocked by treatment for 1 hour in PBS containing 0.1% Tween-20 and 3% BSA. The membranes were then incubated with the polyclonal antibody pabBp19 (1:2000) against porcine pp-vWF$^{14,18}$ and a commercial antibody against human vWF (A0082 Dako, 1:2000) for 1 hour at room temperature and washed several times in PBS/0.1% Tween-20. After incubation with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulins (1:2000), the membranes were washed several times in the same buffer, and the labeled peroxidase activity was developed by the enhanced chemiluminescence method (Amersham). Autoradiog-
raphy was performed at room temperature with the use of Agfa Curix RP2 films.

Ex Vivo and In Vitro Platelet Aggregation

Platelet aggregation was performed in platelet-rich plasma (PRP) as previously reported. PRP count was adjusted to 300,000/mL by dilution with platelet-poor plasma. ADP (2, 5, and 10 μmol/L) and collagen (5, 10, and 20 μmol/L) were used as aggregating agents, and the dose-response curves were registered with a strip-chart recorder. Platelet aggregation was also performed in vitro with botrocetin (5 μg/mL). Atorvastatin, a lipophilic statin, was dissolved in dimethyl sulfoxide, and graded doses of the drug (10⁻³ to 10⁻⁴ mol/L) were incubated with PRP for 10 minutes. Extent of aggregation was calculated from the maximum change in light transmission measured in percent.

Statistical Analysis

All values are expressed as mean±SEM, unless otherwise stated. Differences between groups were evaluated by the unpaired 2-tailed t test. Values of P<0.05 were regarded as statistically significant.

Results

Platelet Deposition Induced by a Triggering Vessel Wall

Platelet deposition triggered by vessels with erosion at a high shear rate was significantly reduced in the A-treated animals (16.7±1.8×10⁶ versus 33.3±5.8×10⁶/cm² in C, a 50% reduction; P<0.01, Figure 1). Platelet deposition was not affected by treatment when the eroded vessel was perfused at low shear rate or when a disrupted, severely damaged vessel wall was perfused at either high or low shear rate (Figure 1).

Immunohistochemistry of Perfused Vessel Wall

Figure 2 is a representative view of the deposition of fibrin (green) and platelets (red) over subendothelium (Figure 2A and 2B) and the medial layer (Figure 2C and 2D), which were perfused in the chamber at high (1690 s⁻¹; Figure 2A and 2C)
and low (212 s\(^{-1}\); Figure 2B and 2D) shear rates. Platelet adhesion was the prevalent mechanism of platelet deposition over the eroded vessel wall (subendothelium), whereas platelets were aggregated over the severely damaged vessel wall, in which a layer of fibrin appeared between the vessel wall and the platelets.

**Characterization of Arterial Lesions**

Feeding of the pigs with the hypercholesterolemic diet for 8 weeks induced thickening of the intima in the coronary arteries (Figure 3). This thickening was induced to a lesser extent in the abdominal aorta, but it did not appear in the thoracic aorta. Atorvastatin reduced development of the lesions in the coronary arteries, as determined by a reduction in intimal area (Figure 4A: C, 1.50±1.25 versus A, 0.26±0.31 mm\(^2\)), I/M ratio (Figure 4C: C, 0.47±0.26 versus A, 0.05±0.02), and percent stenosis (Figure 4D: C, 31±12% versus A, 4±4%).

**Platelet Aggregation**

Ex vivo platelet aggregation (either collagen or ADP induced) in PRP was similar in A-treated animals and C animals. In vitro platelet aggregation (botrocetin induced) was not altered when graded concentrations of atorvastatin were incubated with PRP (data not shown).

**Plasma Lipids**

Plasma total cholesterol (the Table) was reduced by 28% in A-treated animals versus C. This reduction was attributed mainly to the decline in the VLDL cholesterol fraction, as it was reduced by 62% in A-treated animals. Hematological values were not altered in A-treated animals; hence, reductions in platelet deposition were not due to a diminished red blood cell count or hematocrit.

**Platelet and Plasma vWF**

Levels of vWF were determined by ELISA in plasma and in soluble and membrane platelet fractions. vWF levels were not modified by atorvastatin treatment in either of the biophases (plasma, in IU/dL, or platelet subfractions, in IU/100 mg protein) evaluated (C: plasma, 25.6±8.7; membrane platelet fraction, 8.5±3.0; and soluble platelet fraction, 2.4±1.9; versus A: plasma, 29.1±10.9; membrane platelet fraction, 7.5±0.7; and soluble platelet fraction, 2.1±0.5). Platelet soluble and membrane fractions were also analyzed by Western blotting for the presence of vWF and pp-vWF. Differences were not found between platelets from treated and placebo animals (Figure 5).

**Discussion**

We have shown in this study that induction of atherosclerosis with diet and simultaneous treatment with atorvastatin re-
duces platelet deposition at a high shear rate, which mimics conditions existing in stenotic vessels. However, this reduction was dependent on the type of lesion in the triggering vessel: atorvastatin was effective in reducing platelet deposition on mildly damaged, but not on severely damaged, vessel walls. We have also shown previously that atorvastatin reduced platelet deposition in a rabbit model of combined dyslipemia under the same flow conditions, ie, at 1690 s\(^{-1}\) shear rate and on a mildly damaged vessel wall.\(^7\)

On the other hand, ex vivo platelet aggregation induced by ADP or collagen was not altered by the treatment, indicating that the platelet glycoprotein (GP) IIb/IIIa receptor was not affected by atorvastatin. To evaluate whether atorvastatin could directly modulate the function of the platelet GP Ib receptor, we incubated increasing concentrations of atorvastatin with PRP and studied botrocetin-induced aggregation,\(^20\) without finding any modification. vWF binding to the platelet GP Ib receptor is the mechanism proposed for platelet interaction with the subendothelium at high shear rates.\(^21\) Also in pigs, vWF mediates the interaction of platelets with eroded vessels at high shear rates.\(^22,23\) Platelet interaction with a severely damaged vessel wall involves aggregation. As shown by immunohistochemical analysis, platelets were aggregated over severely damaged vessel walls, whereas adhesion was the prevalent mechanism for platelet deposition over eroded vessel walls. Therefore, we investigated whether vWF was altered in platelets and/or plasma and found that their concentrations were unaltered by treatment with atorvastatin.

**Plasma Lipids and Hematological Values in Control and Atorvastatin-Treated Pigs**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>A-Treated</th>
<th>% Lipid Reduction in A vs C</th>
<th>n (Animals per Group)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Cholesterol</strong></td>
<td>381±53</td>
<td>276±44 (28)</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td><strong>HDL Cholesterol</strong></td>
<td>59±10</td>
<td>59±7</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td><strong>LDL Cholesterol</strong></td>
<td>230±33</td>
<td>211±35 (9)</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td><strong>VLDL Cholesterol</strong></td>
<td>74±26</td>
<td>28±10 (62)</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td><strong>RBCs, (\times 10^{12}/\mu L)</strong></td>
<td>5.4±0.3</td>
<td>5.2±1.4</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td><strong>PLT, (\times 10^{12}/\mu L)</strong></td>
<td>430±44</td>
<td>503±75</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td><strong>Hct, %</strong></td>
<td>27.2±0.2</td>
<td>25.2±5.2</td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

RBCs indicates red blood cells; PLT, platelet count; and Hct, hematocrit. Results are expressed as mean±SD (% lipid reduction in A vs C). Total cholesterol was reduced by 28% in A-treated animals, mainly due to a reduction in the VLDL fraction. n=3 per group.

**Figure 4.** Effect of treatment with atorvastatin on the development of intimal thickening. Vessels were excised, cut, and stained using Masson’s trichrome technique. A, intimal area; B, medial area; C, intima/media ratio; and D, % stenosis. Thickening was markedly diminished by atorvastatin in coronary arteries only (black bars), whereas it had no effect on the other vessels (thoracic aorta, white bars; abdominal aorta, grey bars). n=3 animals per group.
operation of those lesions in the right coronary arteries. Other arteries developed only a mild intimal thickening. The atorva-
statin dose used in this study has been previously shown to
diminish cholesterol plasma concentration by a mechanism
related to a decrease of VLDL and LDL apolipoprotein B
production in miniature pigs.24 In our study, cholesterol reduc-
tion was attributed mainly to a strong reduction in the VLDL
fraction, which is consistent with previous work.

In summary, this study indicates that atorvastatin can posi-
tively contribute to slowing the progression of cardiovascular
disease, both by attenuating coronary stenotic lesion develop-
ment and by reducing platelet reactivity to eroded vessels.

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decreases both VLDL and LDL apolipoprotein B production in miniature

Figure 5. Western blot analysis of pp-vWF and vWF. Twenty micrograms each of soluble (S) and membrane (M) fractions were
obtained from isolated pig platelets as described in Methods. NL indicates normolipemic animals, as diet controls; PHP3 and PHP4,
hyperlipemic animals; and PHA3 and PHA4, A-treated animals. Samples were electrophoresed on 7.5% SDS-polyacrylamide gels under
reduced conditions and transferred to nitrocellulose filters. Primary polyclonal antibodies were pabBp19 directed against platelet por-
cine pp-vWF and Dako A0082, against human vWF, which cross-reacts with platelet porcine vWF (both simultaneously at a dilution of
1:2000). The secondary antibody was horseradish peroxidase–conjugated goat anti-rabbit immunoglobulins (Dako P-448, 1:2000).
Reaction was visualized by chemiluminescence. Arrow shows immunolabeling of porcine platelet pp-vWF (97 kDa).
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