L-4F Alters Hyperlipidemic (But Not Healthy) Mouse Plasma to Reduce Platelet Aggregation

Georgette M. Buga; Mohamad Navab; Satoshi Imaizumi; Srinivasa T. Reddy; Babak Yekta; Greg Hough; Shawn Chanslor; G.M. Anantharamaiah; Alan M. Fogelman

Background and Purpose—Hyperlipidemia is associated with platelet hyperreactivity. We hypothesized that L-4F, an apolipoprotein A-I mimetic peptide, would inhibit platelet aggregation in hyperlipidemic mice.

Methods and Results—Injecting L-4F into apolipoprotein E (apoE)–null and low-density lipoprotein receptor–null mice resulted in a significant reduction in platelet aggregation in response to agonists; however, there was no reduction in platelet aggregation after injection of L-4F into wild-type (WT) mice. Consistent with these results, injection of L-4F into apoE-null mice prolonged bleeding time; the same result was not found in WT mice. Incubating L-4F in vitro with apoE-null platelet-rich plasma also resulted in decreased platelet aggregation. However, incubating washed platelets from either apoE-null or WT mice with L-4F did not alter aggregation. Compared with WT mice, unstimulated platelets from apoE-null mice contained significantly more 12-hydroxy 5,8,10,14-eicosatetraenoic acid, thromboxane A 2, and prostaglandins D 2 and E 2. In response to agonists, platelets from L-4F–treated apoE-null mice formed significantly less thromboxane A 2, prostaglandins D 2 and E 2, and 12-hydroxy 5,8,10,14-eicosatetraenoic acid.

Conclusion—By binding plasma-oxidized lipids that cause platelet hyperreactivity in hyperlipidemic mice, L-4F decreases platelet aggregation. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: Platelets ■ apoA-I mimic peptides ■ L-4F ■ Arachidonic acid metabolism ■ apoE null mice

Hyperlipidemia is a risk factor associated with oxidative stress, the generation of oxidized lipoproteins, platelet hyperreactivity, and thrombogenic potential. Increasing evidence indicates that interactions between platelets and oxidized lipoproteins play a major role in the initiation, development, and progression of atherosclerosis. Apolipoprotein A-I (ApoA-I), apoA-I Milano, or high-density lipoprotein (HDL) has been shown to inhibit platelet hyperreactivity and reverse the prothrombotic effects of hyperlipidemia. The atheroprotective and anti-thrombotic activity of HDL is generally attributed to the ability of HDL to promote reverse cholesterol transport and to the antioxidant and anti-inflammatory properties of the lipids and proteins associated with HDL. We recently reported that the apoA-I mimetic 4F peptides bind oxidized lipids with much higher affinity than apoA-I, thus explaining their ability to be effective in animal models and in preliminary human studies in vivo or in vitro at very low concentrations.

Patients with hyperlipoproteinemia show enhanced platelet reactivity, increased production of the arachidonic acid (AA) metabolites thromboxane A 2 (TXA 2) and 12-hydroxyeicosatetraenoic acid (12-HETE), secretion of mediators from platelet-dense granules, and a reduction of prostacyclin receptors on the platelet membrane that might prevent the antiplatelet action of prostacyclin.

Based on the reported evidence on the role of HDL in platelet activation in hyperlipidemia and the knowledge that hyperlipidemic mice have elevated levels of oxidized lipids, we hypothesized that L-4F would exert an inhibitory effect on increased platelet aggregability in hyperlipidemic mice.

Methods

The apoA-I mimic peptide L-4F was synthesized as previously described. Supplemental data (available at: http://atvb.ahajournals.org) describe other materials used in this study. Data are given as mean±SEM unless otherwise indicated.

Mice

Details on the mice used in these studies can also be found in the supplemental data. The mice were injected subcutaneously with...
L-4F at doses ranging between 0.01 and 1.00 mg/kg per day in 0.1 mL of vehicle (ABCT) containing 50-mmol/L ammonium bicarbonate, pH 7.0, and 0.1-mg/mL Tween-20 or vehicle only for various time periods; one hour after the last injection, blood was collected. All animal procedures were approved by the University of California, Los Angeles, Animal Research Committee.

Platelet Preparation and Aggregation
Blood was collected in 3.8% sodium citrate (9:1 vol/vol) from the retro-orbital plexus of isoflurane-anesthetized mice using heparin-coated capillary tubes. Blood from 8 to 10 mice was pooled for the experiments. The citrated blood was centrifuged at 150g for 10 minutes at room temperature to obtain platelet-rich plasma (PRP) and centrifuged again at 1800g for 15 minutes to obtain platelet-poor plasma. Contaminating red blood cells and leukocytes in the PRP were removed by a two-minute centrifugation at 180g. The number of platelets in PRP was counted manually by light microscopy using a hemocytometer at a magnification of ×400. Five PRP samples were counted by one investigator and recounted by two additional observers blinded to treatment. The coefficient of variation for interobserver measurements was 12%±1%. The platelet number in the PRP was adjusted to 1×10⁶ to 5×10⁷ cells per milliliter, with platelet-poor plasma as a diluent. For the preparation of washed platelets, blood collected in acid-citrate-dextrose was processed as described without any modifications. Briefly, PRP containing protaglandin E1, 1 μg/mL, to prevent activation during washing was sedimented by centrifugation at 1800g for ten minutes and gently washed twice with the platelet wash buffer (pH 6.5). After centrifugation, the platelet pellet was resuspended in a modified calcium-free HEPES-Tyrode buffer (pH 7.4) and diluted to the final concentration of 1×10⁶ to 3×10⁷ platelets per milliliter. Platelets suspended in either plasma or HEPES-Tyrode buffer were incubated at room temperature for 30 minutes with gentle agitation. Murine fibriogen, 1 mg/mL, and calcium chloride, 1 mmol/L, were added to the washed platelet suspension 30 seconds before agonist addition. Platelet aggregation was conducted at 37°C as a constant stirring rate of 1000 rpm in a four-channel profiler (PAP-4 Platelet Aggregation Profiler; Bio/Data Corporation, Horsham, Pa). Aggregation of platelets was elicited by the addition of the following agonists: adenosine diphosphate (ADP), 0.5 to 20.0 μM, collagen, 0.048 to 0.190 mg/mL; and AA, 25 to 500 μg/mL. Suboptimal concentrations of agonists were used after establishing the concentrations that caused minimal and maximal aggregation for each experiment. All experiments were repeated at least three times using platelets from different mice. The resulting aggregation was measured as the change in light transmission, was recorded until a plateau was reached. The following platelet aggregation parameters were used to evaluate the effects of L-4F on platelets: amplitude or the maximum sustained rate of aggregation (Vₘₐₓ) and Vₘₐₓ (ie, the maximum sustained rate of aggregation) determined from the steepest slope of the aggregation curve and expressed as a change in the optical density per second; and lag time (for PRP only), representing the elapsed time (in seconds) between agonist addition and the start of aggregation. For in vitro studies of L-4F (0.01, 0.10, and 1.00 mg/mL) or vehicle alone, was added to PRP or to washed platelets and incubated for 60 minutes at 37°C with gentle stirring in the aggregometer; platelet aggregation was determined in response to the indicated agonists.

Measurement of TXA₂ Formation
Mice were injected subcutaneously with L-4F at a dose of 1 mg/kg per day in 0.1 mL of vehicle (ABCT) or vehicle only on day one and were injected twice more every 24 hours. One hour after the last injection, blood was collected for platelet preparation and aggregation studies. The formation of TXA₂ in PRP containing 1×10⁶ platelets per milliliter from L-4F– or vehicle-injected mice was measured by determining thromboxane B₂ (TXB₂), the stable metabolite of TXA₂ in the samples used for platelet aggregation in the presence or absence of the cyclooxygenase (COX) 1 inhibitor, SC-560 (1 μmol/L), which was added in dimethyl sulfoxide and preincubated with the PRP for 30 minutes at room temperature. The reaction initiated by the addition of ADP (20 μmol/L), collagen (0.19 mg/mL), or AA (500 μg/mL) for 5 minutes at 37°C at a stirring rate of 1000 rpm was terminated by the addition of 80 μmol/L of aspirin and 10 mmol/L of EDTA, followed by rapid freezing and storing at −80°C. The amount of TXB₂ in medium was determined by using the TXB₂ enzyme immunoassay kit, according to the procedure described by the manufacturer (Assay Designs, Inc, Ann Arbor, Mich).

Other Procedures
LC-MS/MS analysis is described in the supplemental material (available at: http://atvb.ahajournals.org). Mice were injected subcutaneously with L-4F at a dose of 1 mg/kg per day in 0.1 mL of vehicle or vehicle only on day one and were injected twice more every 24 hours. One hour after the last injection, tail bleeding time was measured in isoflurane-anesthetized mice. Aspirin, 10 mg/kg, administered by stomach gavage 24 hours before bleeding, was used as a positive control. For details of the method used to measure tail bleeding time, please see the supplemental material (available at: http://atvb.ahajournals.org).

Statistical Analyses
For methods used for statistical analyses, please see supplemental material (available at: http://atvb.ahajournals.org). Differences were considered statistically significant at P<0.05 or less.

Results
L-4F Inhibits Agonist-Induced Platelet Aggregation Ex Vivo in Apolipoprotein E– and Low-Density Lipoprotein Receptor–Null Mice
L-4F (0.01, 0.10, and 1.00 mg/kg) injected subcutaneously dose dependently inhibited the ex vivo aggregation of platelets (Figure 1) stimulated as follows: (1) ADP (concentration of dose that produces 50% inhibition [IC₅₀] values of 2.60, 6.69, 7.32*, and 7.56* μM for the administration of ABCT or L-4F at doses of 0.01, 0.10, and 1.00 mg/kg, respectively; *P<0.05 and **P<0.01); (2) collagen (IC₅₀ values of 0.0035, 0.0130, 0.0490, and 0.0820* μg/mL, respectively); (3) AA with IC₅₀ values of 91.4, 95.6, 551**, and 685** μg/mL, respectively. In addition, L-4F significantly reduced the slope and increased the lag time in response to AA, collagen, and ADP (data not shown).

Similar results were obtained when L-4F (but not vehicle) was injected into low-density lipoprotein receptor (LDLR)–null mice fed an atherogenic diet. Because of the marked hyperlipidemia associated with turbid plasma that was induced in these mice, it was necessary to use washed platelet suspensions rather than PRP. Aggregation elicited by collagen, 0.096 mg/mL, was significantly decreased (P=0.003) in the washed platelet suspensions from L-4F–injected LDLR-null mice (72.00%±3.61% aggregation) but not in those from vehicle-injected LDLR-null mice (101.33%±2.85% aggregation). AA (250 μg/mL) induced aggregation was also significantly inhibited (P=0.006) in the washed platelet suspensions from L-4F–injected LDLR-null mice.
LDLR-null mice (52.33% ± 5.89% aggregation) but was not significantly inhibited in the platelets from vehicle-injected LDLR-null mice (89.33% ± 3.67% aggregation).

**L-4F Inhibits Agonist-Induced Platelet Aggregation In Vitro in the Presence of Plasma From Apolipoprotein E–Null Mice But Not in the Absence of Plasma**

The incubation of PRP from apolipoprotein E (apoE)–null mice with L-4F, 500 ng/mL (but not vehicle), significantly reduced platelet aggregation in response to ADP (Figure 2, panel A) and collagen (Figure 2, panel B). In contrast to these results, adding L-4F in vitro to washed platelets from either apoE-null (Figure 3, panels A–C) or wild-type (WT) mice did not inhibit platelet aggregation (Figure 3, panels D–F).

These results indicate that L-4F does not have a direct effect on platelets but acts through some component of plasma that influences platelet function. As previously reported, there was no change in plasma total cholesterol, triglycerides, HDL cholesterol, or apolipoprotein B–containing cholesterol levels after the injection of L-4F in these experiments (data not shown).

A single subcutaneous injection of L-4F in apoE-null mice significantly inhibited platelet aggregation in response to ADP, collagen, or AA for up to 72 hours after injection (data not shown). Ninety-six hours after injection, there was no significant difference in platelet aggregation between mice injected with L-4F or vehicle alone in response to any of the agonists (data not shown).

Although a single subcutaneous injection of L-4F, 0.01 mg/kg per day, was without a significant effect (Figure 1), the administration of the peptide by Alzet osmotic pumps delivering L-4F at a dose of 0.01 mg/kg per day for two weeks significantly reduced platelet aggregation in response to ADP, collagen, or AA compared with mice implanted with pumps delivering the same amount of vehicle without L-4F (data not shown).

**L-4F Inhibits Agonist-Induced TXB₂ Production**

TXB₂ is the stable hydrolysate of TXA₂, which is generated from the AA released from platelet plasma membranes after the sequential activities of the cytosolic phospholipase A₂–COX-1–TXA₂ synthase pathway. After treatment with L-4F, platelet stimulation with AA resulted in significantly less TXB₂ production (approximately 30% less) (data not shown). Under these conditions in the same samples, aggregation was reduced by more than 40% (data not shown). The addition of the COX-1 inhibitor SC-560 further inhibited platelet TXB₂ formation.

**Figure 1.** Apolipoprotein E-null mice were injected subcutaneously with L-4F, 0.01, 0.10, and 1.00 mg/kg, or with vehicle (ABCT) for 48 hours. One hour after the last dose, the mice hemorrhaged, platelet-rich plasma was prepared, and the percentage of platelet aggregation in response to increasing concentrations of adenosine diphosphate (ADP) (A), collagen (B), and arachidonic acid (AA) (C) was determined, as described in the “Methods” section. Blood from 8 to 10 mice was pooled for each experiment. Data are given as mean ± SEM (n = 5). *P < 0.05 and **P < 0.01.

**Figure 2.** L-4F or vehicle alone was added to platelet-rich plasma containing 3 × 10⁸ platelets per milliliter from untreated apolipoprotein E–null mice and incubated, and platelet aggregation was determined in response to adenosine diphosphate (ADP) (A) and collagen (B), as described in the “Methods” section. Blood from 8 to 10 mice was pooled for each experiment. Data are given as mean ± SEM from three different experiments and are expressed as the percentage of platelets aggregating. *P < 0.05, **P < 0.01, and ***P < 0.001.
and aggregation in platelets taken from both vehicle- and L-4F–treated mice (data not shown). Similarly, the slope exhibited significant reductions that were comparable with the reduction in TXB2 production (data not shown).

Conversely, the lag times were increased in the platelets taken from the L-4F–treated mice vs vehicle controls. The concentrations obtained in platelets taken from the L-4F–injected mice were expressed as percentage of platelets calculated as nanograms

Table 1. **p**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>12-HETE</th>
<th>TXB2</th>
<th>PGD2</th>
<th>PGE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen, (0.05 mg/mL (n=7))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>58.7±5.4</td>
<td>22.2±11.7</td>
<td>27.8±7.1</td>
<td>28.8±10.5</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ADP, (20 μmol/L) (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>75.8±3.3</td>
<td>76.2±12.1</td>
<td>83.4±5.0</td>
<td>57.4±8.9</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A23187, (2.5 μmol/L (n=4))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>53.9±17.7</td>
<td>39.6±20.1</td>
<td>39.1±16.3</td>
<td>44.5±15.3</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Arachidonic acid, (125 μg/mL (n=5))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>73.4±9.4</td>
<td>67.4±16.8</td>
<td>64.2±15.3</td>
<td>69.4±16.7</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U46619, 1 μmol/L (n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>95.7±9.2</td>
<td>93.0±21.2</td>
<td>104.7±25.8</td>
<td>77.3±19.0</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

L-4F Treatment Inhibits Agonist-Induced Formation of TXB2, Prostaglandins D2 and E2, and 12-HETE Without Changing the Concentration of Plasma Lipids

In addition to TXB2, a number of other eicosanoids derived from the AA cascade via COX-1 and 12-lipoxygenase enzymatic pathways were analyzed by LC-MS/MS. PRP obtained from L-4F– or vehicle-treated mice was stimulated with the following agonists: collagen (0.05 mg/mL), ADP (20 μmol/L), AA (125 μg/mL), calcium ionophore A23187 (2.5 μmol/L), and the TXA2 mimetic (U46619) (1 μmol/L).24

As shown in Table 1, except for U46619, which directly activates platelet TXA2 receptors, the production of AA metabolites 12-HETE, TXB2, and prostaglandins D2 (PGD2) and E2 (PGE2) was significantly diminished in platelets from L-4F–treated mice vs vehicle controls. The rank order for AA metabolites (calculated as nanograms

Figure 3. Washed platelets (3×10⁸ platelets per milliliter) from untreated apolipoprotein E–null mice were prepared as described in the “Methods” section and incubated for 60 minutes under gentle agitation with L-4F, 1 μg/mL, or vehicle. Platelet aggregation was determined in response to adenosine diphosphate (ADP) (A), collagen (B), and arachidonic acid (AA) (C), as described in the “Methods” section. Washed platelets (3×10⁸ platelets per milliliter) from untreated type C57BL/6 mice were prepared as described in the “Methods” section and incubated for 60 minutes under gentle agitation with L-4F, 0.01, 0.10, and 1.00 μg/mL, or vehicle. Platelet aggregation was determined in response to ADP (D), collagen (E), and AA (F), as described in the “Methods” section. Blood from 8 to 10 mice was pooled for each experiment. Data are shown as mean±SEM from three different experiments and are expressed as the percentage of platelets aggregating. ABCT indicates.
per 3×10^8 platelets) was 12-HETE > TXA2 > PGE2 > PGD2; the rank order for AA metabolites (calculated as a percentage of the most abundant metabolite) was 12-HETE (100%) > TXA2 (12%) > PGE2 (0.6%) > PGD2 (0.3%). In stimulated platelet suspensions preincubated with SC-560, the production of TXB2 was significantly reduced in an agonist-dependent manner with the potency rank order of AA > collagen > A23187 > U46619 > ADP, and was associated with a marked increase of 12-HETE formation (Table 2), suggesting that in the presence of COX-1 inhibitor the AA is diverted toward the 12-lypoxigenase pathway to generate additional 12-HETE.25

### Table 2. ●●●*

<table>
<thead>
<tr>
<th>Agonist</th>
<th>% of DMSO When SC-560 is Added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TXB2 Vehicle</td>
</tr>
<tr>
<td>Collagen, 0.05 mg/mL (n=3)†</td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>2.3±0.7</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ADP, 20 μmol/L (n=4)†</td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>56.8±15.8</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
</tr>
<tr>
<td>A23187, 2.5 μmol/L (n=3)†</td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>6.3±0.9</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Arachidonic acid, 125 μg/mL (n=3)†</td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>1.0±0.0</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>U46619, 1 μmol/L (n=6)†</td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>25.2±1.7</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Data in parentheses indicate number of experiments.

### AA Metabolite Levels Are Higher in Platelets From ApoE-Null Mice Than in Platelets From WT Mice

Unstimulated platelets from apoE-null mice contained significantly more 12-HETE, TXB2, PGD2, and PGE2 than unstimulated platelets from WT C57BL/6 mice (Table 3).

### L-4F Treatment Results in Prolonged Bleeding Time in ApoE-Null Mice But Not in WT Mice

The treatment of apoE-null mice with L-4F resulted in a significant (P<0.001) increase in the bleeding time compared with vehicle-treated mice (3.76±0.73 vs 1.68±0.25

### Table 3. ●●●*  

<table>
<thead>
<tr>
<th>Unstimulated platelets, ng/3×10^8</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 (n=3)</td>
</tr>
<tr>
<td>12-HETE TXB2 PGD2 PGE2</td>
</tr>
<tr>
<td>512.00±4.04</td>
</tr>
<tr>
<td>2050.8±525.6</td>
</tr>
</tbody>
</table>

*Apoe indicates apolipoprotein E; HETE, 12-hydroxy 5,8,10,14-eicosatetraenoic acid; NA, not applicable; PGD2, prostaglandin D2; PGE2, prostaglandin E2; TXB2, thromboxane B2.

*Untreated (ie, no injections) wild-type C57BL/6 and apolipoprotein E (apoE)–null mice hemorrhaged, and blood was pooled from 8 to 10 mice for each experiment. Platelet-rich plasma was prepared as described in the “Methods” section, and the levels of 12-HETE, TXB2, PGD2, and PGE2 in the platelets were determined as described in the supplemental methods. Data are given as mean±SEM unless otherwise indicated. P values indicate the level of significance for the difference between the mean values in the apoE-null platelets compared with the C57BL/6 platelets.

*Approximately 250 mean values are shown.

*P values indicate the level of significance for the difference between the mean values in the apoE-null platelets compared with the C57BL/6 platelets.
minutes). The addition of oral aspirin significantly ($P<0.01$) prolonged the bleeding time of vehicle-treated apoE-null mice (from 1.68±0.25 to 4.65±0.65 minutes). However, in L-4F–treated apoE-null mice, the increase in bleeding time after L-4F treatment alone (3.76±0.73 minutes) was not significantly increased by the addition of aspirin (4.14±0.48 minutes). WT C57BL/6 mice did not show a significant increase in bleeding time with L-4F treatment (2.06±0.24 minutes) compared with vehicle treatment alone (1.84±0.21 minutes). However, the bleeding time in response to aspirin in WT mice increased significantly ($P<0.01$) to 3.93±0.55 minutes. Untreated (ie, no injections) WT C57BL/6 mice had a slightly, but significantly, longer bleeding time (2.10±0.21 minutes) than untreated apoE-null mice (1.62±0.10 minutes) ($P=0.049$).

**Discussion**

In the present study, we investigated the effects of the apoA-I mimetic peptide L-4F on platelet aggregation in WT, LDLR-null, and apoE-null mice on the same genetic background. Because no information regarding the effects of apoA-I mimetic peptides on platelet function was available, we chose to use several physiologically active agonists (collagen, ADP, AA, A23187, and U46619) with distinct platelet activation and aggregation signaling pathways.

The administration of L-4F in vivo significantly reduced ex vivo the percentage of aggregating platelets (Figure 1), accompanied by the expected changes in slope and lag time (data not shown) in response to increasing concentrations of ADP, AA, or collagen compared with vehicle-treated mice. L-4F treatment in vitro of PRP produced similar results; however, incubation of washed platelets with L-4F was without effect, suggesting that L-4F acts on plasma components that modulate platelet function.

The plasma half-life of L-4F after a single subcutaneous injection in mice is on the order of only one hour. The fact that a dose of 0.01 mg/kg of L-4F administered by a single subcutaneous injection was not effective (Figure 1), but that the same dose administered by continuous infusion by Alzet pumps for two weeks was effective (data not shown), suggests that the time during which the peptide is present in plasma may be more important than the peak plasma concentration.

TXA$_2$, one of the major metabolites of AA and a potent endogenous platelet agonist and vasoconstrictor, plays an important role in platelet-thrombus formation. TXA$_2$ is produced in platelets from AA via the COX-TXA$_2$ synthase pathway. The stable hydrolysate of TXA$_2$, TXB$_2$, was significantly reduced in agonist-stimulated platelets obtained from L-4F–treated mice compared with vehicle-treated mice. The inhibition of TXB$_2$ formation paralleled the effects observed for platelet aggregation, suggesting that the inhibition of the COX-TXA$_2$ synthase pathway was at least in part responsible for the reduction in platelet aggregation.

The formation of TXB$_2$, PGD$_2$, PGE$_2$, and 12-HETE was significantly inhibited in platelets obtained from L-4F–injected mice and stimulated with collagen, A23187, ADP, or AA but not with U46619. U46619 addition in the absence of COX-1 inhibition continues the cycle of TXA$_2$ receptor activation–AA liberation–TXA$_2$ formation, generating an excess of TXA$_2$ (Table 1). The inhibition of COX-1 further reduced TXB$_2$ (Table 2) and PDG$_2$ and PGE$_2$ formation (data not shown) and significantly increased 12-HETE accumulation (Table 2), confirming that AA used for both 12-HETE and TXB$_2$ biosynthesis is derived from a single phospholipid pool that can be shunted from one pathway to the other. In addition, the use of U46619 in the presence of COX-1 blockade also demonstrated that L-4F lacks any inhibitory effect on TXA$_2$ synthase activity or on the TXA$_2$ receptor (Table 2).

The decreased platelet aggregation and TXB$_2$ formation after treatment with L-4F was associated with an increased bleeding time in the apoE-null mice but not in the WT C57BL/6 mice. Although the difference in bleeding time between the two treatment groups was statistically significant, no spontaneous bleeding was observed in the L-4F–injected mice and no additive or synergistic effects were observed between aspirin and L-4F. The small differences in the bleeding times in vehicle-treated mice vs untreated mice may indicate a slight effect of the vehicle.

Our results indicate that L-4F decreases the hypersensitivity of platelets to agonist stimulation in hyperlipidemic mice but not in normolipidemic C57BL/6 mice. L-4F does not have a direct effect on platelets but appears to work through a plasma component, as previously noted. Forte et al. reported that the type of oxidized lipids that have been shown to have a particularly high affinity for the 4F peptide are significantly increased in the plasma of apoE-null mice compared with WT mice. Our results are consistent with L-4F binding and the removal of these oxidized lipids from plasma in hyperlipidemic mice, resulting in altered platelet function. The precise identity and mechanism(s) by which such oxidized lipids influence platelet function will require further studies.

**Sources of Funding**

This study was supported in part by grants HL-30568 and HL-34343 from the US Public Health Service; and Laubisch, Castera, and M. K. Grey Funds at the University of California, Los Angeles.

**Disclosure**

Drs Navab, Reddy, Anantharama, and Fogelman are principals in Bruin Pharma; and Dr Fogelman is an officer in Bruin Pharma.

**References**


L-4F Alters Hyperlipidemic (But Not Healthy) Mouse Plasma to Reduce Platelet Aggregation

Georgette M. Buga, Mohamad Navab, Satoshi Imaizumi, Srinivasa T. Reddy, Babak Yekta, Greg Hough, Shawn Chanslor, G. M. Anantharamaiah and Alan M. Fogelman

Arterioscler Thromb Vasc Biol. published online December 3, 2009;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
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/2009/12/03/ATVBAHA.109.200162.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2010/04/27/ATVBAHA.109.200162.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Materials for OnLine Publication Only

Supplemental Materials

Sodium citrate, Tris-buffered saline, ADP, collagen and arachidonic acid (AA) were purchased from (Bio/Data Corporation, Horsham, PA). The cyclooxygenase (COX)-1 specific antagonist SC-560 was purchased from Cayman Chemical (Ann Arbor, MI). TXB2 EIA kit was obtained from Assay Designs, Inc. (Ann Arbor, MI).

Internal standards for LC/MS/MS analysis: (±)12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12(S)-HETE), 9α,11,15S-trihydroxythromb-5Z,13E-dien-1-oic acid (thromboxane B2; TXB2), 9-oxo-11 α, 15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid (Prostaglandin E2; PGE2), 9α,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid (Prostaglandin D2; PGD2), 12(S)-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic-5,6,8,9,11,12,14,15-d8 (12(S)-HETE-d8) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Oasis HLB (1cc/10mg, 30μm) was obtained from Waters Corporation (Milford, MA). All other reagents were HPLC grade from Sigma-Aldrich (St. Louis, MO).

Mice

Female 8-12 months old wild-type (WT) C57BL/6 and ApoE null mice on a C57BL/6 background (Jackson Laboratories, Bar Harbor, ME) were maintained on a chow diet (Ralston Purina; 3.5 % kcal from fat). Female 3-4 month old LDL receptor null mice on a C57BL/6 background (Jackson Laboratories, Bar Harbor, ME) were placed on an
atherogenic diet (Harlan Teklad #TD94059 containing 1.25% cholesterol) for two weeks prior to study.

**Supplemental Methods**

**LC-MS/MS analysis**

After performing platelet aggregation as described above for TXA₂ formation, the 200μL volume of PRP containing 3×10⁸ platelets/mL from L-4F or vehicle injected mice was transferred to a 2mL polypropylene tube, and spiked with 100 μL of internal standard (12(S)-HETE-d₈, 10ng/ml). The mixture was acidified to pH 3.0 using HCl, mixed further by vortexing, and left for 15min on ice for complete acidification and equilibration. The resulting sample was slowly loaded onto a preconditioned 1cc Oasis HLB solid-phase extraction cartridge on a vacuum manifold (Waters). The cartridge was first washed with 1 mL of 5% methanol in water. 12(S)-HETE, TXB₂ and prostaglandins (PGs) were subsequently eluted with 1 mL methanol. The eluate was evaporated to dryness under argon and resuspended in 100 μL of methanol, vortexed for 30s, and centrifuged at 13200 rpm for 20 min at 4°C to remove any precipitate. The resulting supernatants were transferred to autosampler vials and processed for LC-MS/MS analysis. LC-MS/MS was performed using a quadruple mass spectrometer (4000 QTRAP; Applied Biosystems, Foster City, CA) equipped with electrospray ionization (ESI) source. The HPLC system consisted of Agilent 1200 series LC pump equipped with a thermostatic autosampler (Agilent Technologies, Santa Clara, CA). Chromatography was performed using a Luna C-18(2) column (3 μm particle, 150 × 3.0mm; Phenomenex, Torrance, CA) with a security guard cartridge (C-18; Phenomenex) at 40°C. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1%
formic acid in acetonitrile. The autosampler was set at 4°C. The injection volume was 10 μL and the flow rate was controlled at 0.4mL/min. The gradient program was as follows: 0-3 min, linear gradient from 50% to 60% B; 3-18 min, linear gradient from 60-65% B; 18-22 min, linear gradient from 65-100% B; 22-24 min 100% B; 24-26 min, linear gradient from 100% to 50% B; 26-38 min, 50% B. The data acquisitions and instrument control were accomplished using Analyst 1.4.2 software (Applied Biosystems). The analyses were detected by multiple reaction monitoring (MRM) mode with negative ion detection, and the parameter settings used were: ion spray voltage = -4500 V; curtain gas = 25 psi (nitrogen); ion source gas 1 = 45 psi; ion source gas 2 = 55 psi; gas temperature = 450°C. Collision energy, declustering potential, and collision cell exit potential were optimized for each compound to obtain optimum sensitivity. The transitions monitored were mass-to-charge ratio (m/z): m/z 319.1→179.0 for 12-HETE; 369.1→169.1 for TXB2; 351.1→271.2 for PGD2; 351.1→270.9 for PGE2; 327.1→184.0 for 12(S)-HETE-d8.

**Measurement of tail bleeding time**

The tail was cut transversally at exactly 0.6 cm from the tip with a sharp razor blade and was immediately immersed in warm (37°C) isotonic saline. The blood streaming into the saline solution was timed until it stopped. Bleeding time was defined as the time required for arrest of bleeding, and when necessary bleeding was stopped manually at the 8-minute time point to prevent death.

**Statistical Analyses**

Values are presented as mean ± SEM. Statistical comparisons between two groups were performed with a two-tailed Student's t Test with a Welch correction applied if there was
a significant difference in the standard deviations; repeated-measures were analyzed by ANOVA Tukey-Kramer Multiple Comparisons Test, and IC50 was determined by non-linear regression using InStat and Prizm software (Graph Pad, San Diego, USA).