Inhibition of Factor XIIIa in a Canine Model of Coronary Thrombosis: Effect on Reperfusion and Acute Reocclusion After Recombinant Tissue-Type Plasminogen Activator

By Ronald J. Shebuski, Gary R. Sitko, David A. Claremon, Jack J. Baldwin, David C. Remy, and Andrew M. Stern

The effect of inhibition of factor XIIIa with 2-(1-acetonylthio)-5-methylthiazolo[2,3-b]1,3,4-thiadiazo[2,3-b]1,3,4-thiadiaziolium perchlorate (L-722,151) on coronary thrombolysis and reocclusion was studied in an acute dog model of electrically induced coronary thrombosis. L-722,151 (0.1 mg/kg/min intravenously [IV] or placebo was administered 15 minutes before current initiation (150 μA) and for the duration of the experiment (270 minutes). Fifteen minutes after thrombus formation, heparin (300 U/kg, IV) was administered, followed 45 minutes later by recombinant tissue-type plasminogen activator (tPA) (10 μg/kg/min, IV for 90 minutes). Placebo-treated animals thrombosed at 48.9 ± 8.1 minutes (mean ± SEM) and reperfused in response to tPA at 49.1 ± 9.3 minutes. L-722,151 pretreated animals thrombosed at 44.4 ± 9.7 minutes and reperfused in response to tPA at 16.4 ± 2.8 minutes (P < .05 vs vehicle). Furthermore, residual thrombus mass was reduced by L-722,151 from 8.9 ± 1.9 mg in placebo-treated animals to 1.7 ± 0.6 mg (P < .05 vs vehicle). Acute reocclusion occurred in 86% of placebo and in 75% of L-722, 151–treated animals. The incidence of tPA-induced reperfusion in L-722,151–treated dogs was 100% (8 of 8), whereas only 70% (7 of 10) of placebo-treated dogs reperfused. These results demonstrate that pretreatment with L-722,151 hastens reperfusion time threefold and reduces residual thrombus mass. These effects occurred with no change in systemic blood pressure in response to L-722,151. When L-722,151 was administered 15 minutes after thrombus formation in a separate group of dogs (n = 5), no beneficial effect on thrombolysis time or thrombus mass was observed. Thus, the specific factor XIIa catalyzed crosslinking reaction(s), which may determine(s) resistance to plasmin-mediated fibrin degradation, occur(s) rapidly. Inhibition of this crosslinking by pretreatment with L-722,151 promotes tPA-induced thrombolysis.

© 1990 by The American Society of Hematology.

MATERIALS AND METHODS

Canine coronary artery thrombosis model. Mongrel dogs of either sex (9 to 18 kg) were anesthetized with pentobarbital sodium (30 mg/kg IV), intubated, and ventilated with room air at a tidal volume of 20 mL/kg and a frequency of 12 inflations/min (Harvard respirator, Harvard Apparatus, South Natick, MA). The right femoral artery and vein were exposed, and catheters were inserted for monitoring arterial blood pressure (Statham P23 ID pressure transducer, Gould Inc) and administration of drugs, respectively. The left external jugular vein and the left saphenous vein were also exposed and catheters were inserted for continuous infusion of 5% dextrose in saline (1 mL/kg/h) and infusion of drugs, respectively. A left thoracotomy was performed at the fifth intercostal space and the heart was exposed and suspended in a pericardial cradle. A 2-cm section of the left circumflex (LCX) coronary artery was isolated proximal to the first obtuse marginal branch and instrumented proximal to distal as follows: electromagnetic flow probe (model 501),
Protocol. Dogs were assigned randomly to one of three groups. In group I (n = 10), formulation vehicle or placebo (4% dimethylsulfoxide [DMSO] in phosphate-buffered saline [PBS]) was administered 15 minutes before current was delivered to the circumflex coronary artery and for the duration of the experiment (approximately 270 minutes). One hour after thrombus formation, recombinant tPA (10 μg/kg/min) was administered IV for 90 minutes. Group II (n = 8) consisted of animals receiving L-722,151 (0.1 mg/kg/min, IV) 15 minutes before current initiation and for the duration of experiment. One hour after thrombus formation in group II, tPA was administered at a dose of 10 μg/kg/min, IV for 90 minutes. Group III animals (n = 5) received L-722,151 (0.1 mg/kg/min, IV) 15 minutes after spontaneous electrically induced thrombus formation followed 45 minutes later by tPA at 10 μg/kg/min, IV for 90 minutes. Thus, all animals in the study received identical doses of heparin and tPA at the same time intervals in the protocol. All animals were observed for 1 hour after termination of tPA to determine the incidence and time to reocclusion. At the end of the 1-hour observation period, experiments were terminated with KCl.

Determination of residual thrombus mass. Just before termination, the circumflex coronary artery was ligated proximal to the blood flow probe and distal to the Goldblatt clamp. The artery was then excised, slit longitudinally, and pinned out on a piece of styrofoam with 26G needles. The thrombus was visualized, blotted with filter paper to remove excess blood, removed from the lumen of the vessel with forceps under a dissecting microscope (10X), and weighed on a milligram balance.

Criteria used to judge the time to thrombosis, reperfusion, and reocclusion. Criteria were developed to judge the time to thrombosis, reperfusion, and reocclusion in the canine model used in this study. Thrombosis was judged complete when coronary blood flow fell to zero and remained at zero. Some cycling of blood flow occurs as the thrombus forms: however, only complete, sustained occlusion was used to determine time to thrombosis. Five minutes after complete occlusion, the electrical current to the artery was terminated. tPA-induced reperfusion required that coronary artery blood flow be maintained for at least 5 minutes at a flow rate of at least 50% of control flow. The value used for control blood flow was the coronary blood flow immediately before delivering current to the vessel. Reocclusion was defined as zero blood flow after successful tPA-induced reperfusion. In most cases, cyclical flow reduction (CFRs) occur after tPA-induced reperfusion in this model. Thus, the first CFR that goes to zero flow is used as the temporal endpoint for reocclusion. Some animals exhibit CFRs throughout the 1-hour observation period after termination of tPA, whereas other animals exhibit permanent reocclusion that is devoid of periodic and spontaneous CFRs.

Assay for L-722,151 in plasma. Plasma concentrations of L-722,151 were determined with an in vitro assay based on the direct inhibition of FXIIIa. Purified human FXIIIa was activated at 0.2 mg/mL by 5 U/mL human thrombin (Sigma, St Louis, MO) for 10 minutes at 25°C in the presence of 100 mmol/L NaCl, 5 mmol/L CaCl2, 1 mmol/L EDTA, 10% (vol/vol) glycerol, and 50 mmol/L tris HCl, pH 7.5. The activation mixture was quenched by hirudin (100 U/mL; Sigma) and diluted 10-fold by buffer containing all components except thrombin. To 160 μL of this FXIIIa solution was added 5 μL of plasma samples obtained from the dog during L-722,151 administration, or control (preinfusion) plasma samples to which known concentrations of L-722,151 had been added. Eighty-five microliters of a substrate solution containing 0.33% dimethylated casein (Sigma) and 1.67 mmol/L [14C] putrescine (5 mCi/mmol; New England Nuclear) was added. After a 20-minute incubation at 37°C, a 20-μL aliquot was spotted on a Whatman 3-mm filter disc and the covalently bound radioactivity was deter-
Table 1. Effect of Pretreatment With L-722,151 on Coronary Thrombosis, Thrombolysis, and Reocclusion in the Dog Electrolytic Injury Model

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th></th>
<th>Group II</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tPA</td>
<td>(10 μg/kg/min, IV) +</td>
<td>L-722,151</td>
<td>(0.1 mg/kg/min, IV) +</td>
</tr>
<tr>
<td>Time to occlusion (min)</td>
<td>48.9 ± 8.1</td>
<td>44.4 ± 9.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to reperfusion (min)*</td>
<td>49.1 ± 9.3</td>
<td>16.4 ± 2.8‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reocclusion rate (%)†</td>
<td>6/7 (86%)</td>
<td>6/8 (75%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reocclusion time (min)</td>
<td>21.6 ± 6.0</td>
<td>26.2 ± 8.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual thrombus weight (mg)</td>
<td>6.9 ± 1.9</td>
<td>1.7 ± 0.6‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incidence of reperfusion (%)</td>
<td>7/10 (70%)</td>
<td>8/8 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Range (min): group I, 25–90; group II, 9–32.
†Permanent reocclusion: group I, 5 of 6 (83%); group II 3 of 6 (50%).
‡P < .05 versus vehicle by one-sided Student's t-test.

RESULTS

Animals in group I receiving placebo infusion thrombosed in response to electrolytic injury at 49 ± 8 minutes (Table 1). Thrombolysis occurred in this group at 49 ± 9 minutes after the start of tPA infusion (Fig 2), with acute reocclusion occurring 22 ± 6 minutes after successful reperfusion in 6 of 7 animals. The range of tPA-induced reperfusion times for group I was 25 to 90 minutes. Three animals failed to reperfuse after 90 minutes of tPA infusion. The residual thrombus wet weight in group I was 7 ± 2 mg (Table 1).

Group II animals that received L-722,151 before current initiation and for the duration of the experiment thrombosed at 44 ± 10 minutes, which was not significantly different from group I thrombosis time. However, reperfusion occurred at 16 ± 3 minutes after the start of tPA infusion (Fig 2) with a range of 9 to 32 min (Table 1), a statistically significant (P < .05) difference from group I. Acute reocclusion in group II occurred at the same rate and time as group I. However, residual thrombus wet weight was significantly reduced in group II dogs to 2 ± 1 mg. tPA-induced reperfusion occurred in 100% of animals (8 of 8) receiving L-722,151 infusion.

Group III dogs received L-722,151 15 minutes after electrically induced clot formation. The time to reperfusion in these animals was 59 ± 17 minutes after the start of tPA infusion which was very similar to group I dogs (Fig 2). The reocclusion rate and time as well as clot weight and incidence of reperfusion were also similar to group I animals (Table 2). Thus, dogs, not pretreated with L-722,151 before clot formation failed to reperfuse sooner as had been observed in group II pretreated animals.

A hemodynamic analysis on groups I and II was performed for arterial blood pressure and coronary blood flow (Fig 3). L-722,151 had no significant effect on blood pressure when analyzed at four timepoints in the experiment: control (before current initiation), spontaneous occlusion, tPA-induced reperfusion, and just before termination of the experiment. Coronary blood flow was not affected by L-722,151 at reperfusion: however, at termination there was a slightly greater residual flow for L-722,151–treated animals due to the greater number of vehicle-treated group I animals that exhibited permanent reocclusion (Table 1), and thus zero blood flow.

At an IV infusion rate of 0.1 mg/kg/min, a steady-state concentration of 6 μmol/L L-722,151 was achieved within 30
Fig 3. Mean arterial blood pressure and coronary blood flow at control (before drug or initiation of current), at coronary occlusion, at tPA-induced coronary reperfusion, and at the termination of the experiment for dogs in groups I and II. Asterisk indicates that this latter result is consistent with a previously determined incubation (unpublished observations).

![Graph showing blood pressure and blood flow](image)

Table 3. Plasma Concentrations of L-722, 151

<table>
<thead>
<tr>
<th>Time After Initiation of IV Infusion* (min)</th>
<th>L-722, 151 Plasma Concentrations (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.1 ± 0.8</td>
</tr>
<tr>
<td>10</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>15</td>
<td>3.8 ± 1.9</td>
</tr>
<tr>
<td>30</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>60</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>90</td>
<td>5.9 ± 1.0</td>
</tr>
<tr>
<td>120</td>
<td>7.4 ± 1.3</td>
</tr>
<tr>
<td>180</td>
<td>7.7 ± 1.4</td>
</tr>
</tbody>
</table>

*Infusion rate: 0.1 mg/kg/min.
†For five dogs, 1- and 2-hour samples were measured. For two of these dogs determinations at additional times were made. For N = 2 the range is presented, and for N = 5 the standard error is presented.

Discussing the results of this study, indicator that pretreatment with L-722, 151, an inhibitor of FXIIIa, results in a threelfold decrease in thrombolytic time with a concomitant reduction in residual thrombus weight in an in vivo canine model of thrombosis and thrombolysis. This action of L-722,151 is not associated with any change in systemic blood pressure. However, waiting to start L-722, 151 until 15 minutes after thrombus formation completely eliminated the beneficial effect of FXIIIa inhibition on tPA-induced reperfusion time and residual thrombus weight. Thus, the specific FXIIIa catalyzed crosslinking reaction(s), which may determine resistance to plasmin-mediated fibrin degradation, occur(s) rapidly in vivo. The full potential of this approach, therefore, may only be realized during prophylactic therapy in a patient population at high risk of thrombosis, such as that encountered in unstable angina or in patients with repeated and predictable episodes of deep vein thrombosis. On the other hand, it may be argued that patients with unstable angina, treated with an inhibitor of FXIIIa to prevent crosslinking that go on to infarct, may spontaneously lyse in response to their own endogenous tPA or may be more responsive to exogenously administered thrombolytic agents. It does not appear that FXIIIa inhibition prevents reocclusion, which suggests that incorporation of an anti-platelet agent into a thrombolytic protocol may be necessary as well. It would be of interest to combine an FXIIIa inhibitor with an anti-platelet agent to determine if lysis time as well as acute reocclusion could be decreased.

The results from this in vivo study do not address the exact mechanism by which an inhibitor of FXIIIa is decreasing thrombolysis time. As mentioned above, this action could be due to an inhibition of fibrin γ chain dimer formation, fibrin α chain polymer formation, or the incorporation of α2 antiplasmin into the α chain of fibrin. In vitro at 5 μmol/L, L-722,151 γ chain dimer formation is inhibited less than 20%, while α chain polymer formation is 100% inhibited and α3 antiplasmin incorporation is 75% to 80% inhibited.25 In vivo studies (Bush L., Stern A.M., unpublished observations) in a canine model of thrombosis at the same infusion rate of L-722,151 used in these studies demonstrated complete inhibition of α fibrin chain polymer formation with no appreciable inhibition of γ chain dimer formation. α2 antiplasmin incorporation was not determined. These results indicate that γ chain dimer formation is not significantly inhibited by L-722,151 at concentrations achieved in vivo (2 to 10 μmol/L) and that the enhancements in reperfusion rates result from either the inhibition of a polymer formation or α2 antiplasmin incorporation into the clot. However, our present data is insufficient to determine the relative contribution of each of these mechanisms in shortening lysis times. The extremely narrow temporal window would argue in favor of the kinetically more rapid reaction, α2 antiplasmin incorporation, being the most significant mechanism for enhancing lysis rates in vivo. However, if clot penetration of L-722,151 becomes in part limiting this kinetic argument becomes less cogent. In addition, although in vitro static systems the α2 antiplasmin incorporation reaction appears to be the most important,26 in vivo, especially for arterial clots, the mechanical stability generated by α chain polymers22 may become an important factor not realized in vitro.

Regardless of the exact mechanism by which the inhibition of FXIIIa leads to enhanced clot lysis, our studies demon-
strate that FXIIIa is a potentially important target for therapeutic control of thrombosis. The FXIIIa inhibitor used in this study, L-722,151, is a pharmacologic tool and not a candidate for clinical development. However, L-722,151 may serve as a prototype for the development of safe and efficacious FXIIIa inhibitors.

ACKNOWLEDGMENT

The authors thank Robin Carter for preparation of the manuscript. We also acknowledge Dr C. Bradsher of the Department of Chemistry, Duke University, Durham, NC, from whom we received the original sample of the factor XIIIA inhibitor that was resynthesized in our laboratories at Merck & Co, Inc.

REFERENCES

Inhibition of factor Xllla in a canine model of coronary thrombosis: effect on reperfusion and acute reocclusion after recombinant tissue-type plasminogen activator

RJ Shebuski, GR Sitko, DA Claremon, JJ Baldwin, DC Remy and AM Stern