Active Site-Blocked Factor Xa Prevents Thrombus Formation in the Coronary Vasculature in Parallel With Inhibition of Extravascular Coagulation in a Canine Thrombosis Model

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Factor Xa is a central procoagulant enzyme, linking the intrinsic and extrinsic activation mechanisms to the final common pathway of coagulation. To assess its contribution to pathologic thrombosis, studies were performed in a canine coronary thrombosis model. Thrombus formation was initiated by the application of electric current via a needle electrode placed in the lumen of the left circumflex coronary artery. When 50% occlusion of the vessel developed, the current was stopped and animals received an intravenous bolus of either saline, bovine glutamyl-glycinyl-arginy1-factor Xa (Xai), a competitive inhibitor of factor Xa assembly into the prothrombinase complex, Factor X, or heparin. Animals infused with saline or factor X (300 µg/kg) developed total occlusion of the vessel due to a fibrin/platelet thrombus in 70 ± 11 minutes (36 of 38 animals) and 74 ± 13 minutes (8 of 8 animals), respectively. In contrast, infusion of Xai prevented thrombus formation completely at a dose of 300 µg/kg (8 of 8 animals). As the dose of Xai was decreased, its antithrombotic effect was diminished, with a patency rate of only 2 of 6 animals at a dose of 90 µg/kg. Xai at 300 µg/kg prevented the accumulation of 125I-fibrinogen/fibrin at the site of the coronary thrombus by approximately 63% and decreased deposition of 111In-labeled platelets by approximately 57%. Hemostatic parameters of animals infused with Xai demonstrated prolongation of the PT and dose-dependent increased extravascular bleeding tendency. These data indicate that factor Xa has a comparatively important role in thrombus formation and extravascular hemostasis, and contrast with previous results in this same animal model in which XIa selectively prevented clotting in the coronary vasculature.

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was applied (150 μA) to the needle electrode until a 50% increase in blood flow velocity occurs. This has been shown to correlate with a 40% to 50% decrease in cross-sectional area of the lumen due to thrombus formation at the site of placement of the electrode. When the current was turned off, animals received an IV infusion of either saline (8 mL), factor X (300 μg/kg in approximately 8 mL of saline), or heparin (5,000 U as an IV bolus followed by 200 U/kg/min). Where indicated, animals also received 125I-fibrinogen (5 μCi in 1 mL of saline IV) at the time of stopping the current or 111In-labeled platelets as described below. Fibrinogen was purified and radioiodinated as described previously (specific radioactivity of 130 μCi/mg).15,16

111In-labeling of platelets. After completing the instrumentation, blood was collected from the dogs to label platelets with 111In-8-hydroxyquinoline (oxine) as described by Thakur et al.17 Forty-three milliliters of blood was collected into 7 mL of acid-citrate-dextrose, mixed, and centrifuged at 125g for 20 minutes at room temperature. The platelet-rich plasma was recentrifuged at 1,100g for 5 minutes and the platelet-poor plasma removed. The platelet pellet was suspended in a 500 μL of platelet-poor plasma for labeling. 111InCl3 (Amersham Corp, Arlington Heights, IL) was prepared in 0.3 mol/L acetate buffer (pH 5.3), to which 50 μL of oxine in ethanol (1 mg/mL) was added. After 15 minutes, the complex was extracted twice with 2 mL of methylcaine chloride, dried, and the residue dissolved in 50 μL of absolute ethanol. Approximately 82% to 97% of the original radioactivity was recovered. 111In-oxine (250 to 300 μCi) was then added to platelet suspensions for 30 minutes at 37°C, the mixture was centrifuged at 1,100g for 5 minutes to remove the supernatant plasma, and the platelet pellet was resuspended in 2 mL of autologous plasma. The labeling efficiency was in the range of 33% to 88%. The viability of the labeled platelets was assessed in different ways. The responsiveness of radiolabeled platelets to various aggregating agents was tested and compared with the values obtained before labeling (see below). The in vivo survival of labeled platelets was determined by calculating the percentage of administered radioactivity bound to circulating platelets at different time intervals. At 2, 5, 10, 30, 60, and 120 minutes after the administration of 111In-labeled platelets, a 2-mL blood sample was collected, platelets were isolated as described above, and radioactivity was counted in both the platelet pellet and platelet-poor plasma. The percent recovery of the radiolabel, calculated using standard methods,18 was found to be 89% to 95% in the platelet pellet.

Deposition of 125I-fibrinogen/fibrin into coronary vessel segments was studied as previously described.4 In animals infused with 125I-fibrinogen, either at the time of vessel occlusion or 180 minutes after the current was turned off, the circumflex artery was divided into three 2-cm segments: just proximal to the needle electrode insertion site into the vessel lumen, the site of thrombus formation, which corresponded to the position of the needle, and distal to the thrombus. Each segment was weighed, radioactivity was determined, and the counts were normalized according to the weight of the segment. Accumulated radioactivity in the circumflex artery was expressed as a ratio of that measured in a segment of similar length and weight from the uninstrumented left anterior descending coronary artery.

Radiolabeled platelets were reinjected into the dogs 60 minutes before start of the current and allowed to circulate for 60 minutes. At the end of the study (60 minutes after total occlusion or when the vessel remained patent for 180 minutes after stopping the current) the circumflex artery was removed and the 111In-labeled platelet accumulation ratio was determined as described for the 125I-labeled fibrinogen/fibrin accumulation ratio.

Serotonin levels in EDTA-anticoagulated platelet-poor plasma samples (coronary sinus minus left atrial), as an index of in vivo platelet aggregation,13 were determined by a radioenzymatic assay every 20 minutes throughout the experiment. Additionally, the serotonin concentration in formed thrombus was determined to establish a correlation between 111In-labeled platelet accumulation in thrombus and serotonin concentrations present in the thrombus. For histologic comparison of the thrombi formed in the presence of factor Xai with that of thrombi formed under control conditions (saline or factor X), thrombi from the left circumflex artery were carefully and rapidly removed and processed as described previously.13

Assessment of coagulation, bleeding parameters, and factor Xai levels in canine plasma. The prothrombin time (PT) and platelet aggregation studies in response to ADP, collagen, and U46619 (a thromboxane A2 analog; Upjohn, Kalamazoo, MI) were performed on samples of citrated blood (0.4%) from animals receiving saline, factor X, or factor Xai using standard methods. The bleeding tendency at extravascular sites was assessed as described previously using a modified incisional bleeding time in which a uniform 1-cm deep, 5-cm long abdominal wall incision was made, and a preweighed 4 × 4 inch gauze was inserted for 5 minutes. The gauze was then removed, reweighed, and the weight of blood lost determined.

The level of factor X/Xai antigen in dog plasma was determined by a radioimmunassay with rabbit antibody raised to bovine factor Xai by the general method of Suzuki and Thompson for factor IX antigen, as described previously.21 This antibody did not discriminate between bovine factor X and Xai, and showed no cross-reacting material in undiluted dog plasma, indicating that there was no significant recognition of dog factor X at plasma levels. For the assay, heparinized dog plasma samples (from animals infused with bovine factor Xai) were incubated overnight with 125I-bovine factor Xai and rabbit antithrombin factor X IgG. Bound 125I-factor Xai was precipitated by the addition of protein A-bearing, formalin-fixed staphylococci A (IgG-Sep, Enzyme Center, Malden, MA). Factor Xai was radioiodinated by the lactoperoxidase procedure22 using Enzymebeads (Bio-Rad, Richmond, CA). The limit of sensitivity in this assay was 500 ng/mL, which corresponded to 80% binding on the standard curve.

Statistical analysis. Data was analyzed by one-way analysis of variance. The mean ± SD is shown.

RESULTS

Time to coronary occlusion and histopathology of the thrombus formed. Coronary thrombosis was initiated by application of electric current to the left circumflex coronary artery until an approximately 50% decrease in the cross-sectional area occurred due to thrombus formation, as described previously.8,13 The current was then discontinued, and 70 ± 11 minutes later complete cessation of blood flow occurred (Table 1) due to continuing thrombus formation.8,13 The hemodynamic parameters observed in animals receiving a single IV bolus of the zymogen, factor X (300 μg/kg), at the time the current was shut off, showed the same pattern previously seen in control animals infused with saline8,13; occlusion developed by 74 ± 13 minutes and was accompanied by decreased contractility of the posterior wall (Table 1 and Fig 1, top panel). Administration of factor Xai (300 μg/kg), in place of factor X, prevented occlusion of the vessel (Fig 1, bottom panel, and Table 1). Although coronary blood flow appeared to decrease initially in animals treated with factor Xai, it returned to preocclusion levels by 60 to 90 minutes. Consistent with this observation, hemodynamic parameters and cardiac contractile function (arterial pressure, heart rate, an-
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The inhibitory effect of factor Xai on coronary thrombosis was dependent on the concentration of active site-blocked factor Xa infused (Table 1). Although factor Xai completely prevented occlusive thrombosis when infused at a dose of 300 µg/kg (8 of 8 animals), as the dose was lowered its efficacy declined; the patency rate decreased to about 62%, 40%, and 33% at doses of 150, 120, and 90 µg/kg, respectively. In contrast, no protective effect (0 of 8) was ever observed when high concentrations of factor Xai were infused.

Consistent with these functional data, histologic study of the left circumflex artery from animals infused with factor Xai (300 µg/kg) (Fig 1, bottom panel).

To assess the clearance of bovine factor Xai infused into dogs, bovine Xai antigen was assayed in the plasma of animals that had received a bolus of Xai. A species specific antigen assay for bovine factor Xai demonstrated a rapid increase in antigen level to approximately 9 µg/mL immediately after the infusion that declined to approximately 2 µg/mL by 120 minutes later, and subsequently decayed to the baseline by 180 minutes (data not shown). Bovine factor Xai antigen levels were not significantly different between the blood samples obtained from the left atrium and coronary sinus.

Fibrinogen accumulation in thrombus. In view of the capacity of factor Xai to inhibit factor Xa assembly into the prothrombinase complex,3,6 thereby preventing prothrombin activation, and, ultimately, fibrin formation, its effect on the deposition of 125I-fibrinogen/fibrin in the thrombosed coronary segment was studied (Fig 3A). Vessel segments from animals infused with 125I-fibrinogen, starting at the time the current was discontinued, were used to define a 125I-fibrinogen/fibrin accumulation ratio: the ratio of radioactivity deposited per milligram tissue in the circumflex artery at the site the needle electrode was placed (thrombosed site), or proximal or distal to this area, compared with radioactivity deposited in the uninstrumented left anterior descending coronary artery. In the presence of Xai (300 µg/kg), the 125I-fibrinogen/fibrin accumulation ratio decreased in the thrombosed and distal segments by approximately 63% and 61%, respectively. There was no significant difference in fibrinogen/fibrin accumulation ratios in the circumflex segment proximal to the lesion.

Platelet accumulation in thrombus. Because platelets are an important component of the clot in the electrically induced coronary thrombosis model and thrombin-induced platelet aggregation is thought to contribute significantly to this process, it was important to determine the effect of factor Xai on their contribution to intravascular clotting in this setting (Fig 3B). To compare platelet deposition in the thrombosed, proximal, and distal segments of the left circumflex artery, a platelet accumulation index, analogous to the 125I-fibrinogen/fibrin accumulation ratio above, was defined. To verify the viability of labeled platelets, we determined the 111In counts in blood as a function of time after injection of labeled platelets into the dogs. Two minutes after injection, 95% of the injected counts were present; at 5 minutes, 78%; 10 minutes, 64%; and at 30 minutes, 62%. No significant change in 111In activity was noted after 30 minutes (this circulating radioactivity was more than 89% to 95% associated with the platelets). This strongly suggest that the damaged platelets are rapidly removed by spleen or other reticuloendothelial cells and the remaining circulating 111In was localized in normal platelets. In keeping with this, these platelets demonstrated normal aggregation profiles to the agonists tested. Using 111In-labeled platelets infused into animals at the time the current was discontinued, the platelet deposition indices decreased by approximately 57% and 52% in the thrombosed and distal circumflex artery. There was no significant change in the low platelet deposition index observed in the proximal circumflex. These data, which indicated that factor Xai also caused a significant decrease in platelet deposition into the thrombus, were complemented by the results of peak coronary sinus serotonin levels (Fig 4A), measured as an estimate of platelet activation/release in the circumflex artery and serotonin concentrations in formed thrombus (Fig 4B). In the presence of factor Xai, serotonin concentrations in formed thrombus were reduced by approximately 46%, whereas the peak coronary sinus serotonin levels were decreased by approximately 55%.

Table 1. Effect of Factors X and Xai on Coronary Occlusion in the Electrical Current-Induced Thrombosis Model

<table>
<thead>
<tr>
<th>N</th>
<th>Agent</th>
<th>Dose (µg/kg)</th>
<th>Patency</th>
<th>Time to Occlusion (min) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>Saline</td>
<td>—</td>
<td>0/36</td>
<td>70 ± 11</td>
</tr>
<tr>
<td>8</td>
<td>X</td>
<td>300</td>
<td>8/8</td>
<td>&gt;180*</td>
</tr>
<tr>
<td>8</td>
<td>Xai</td>
<td>150</td>
<td>5/8</td>
<td>152 ± 231†</td>
</tr>
<tr>
<td>7</td>
<td>Xai</td>
<td>120</td>
<td>3/7</td>
<td>121 ± 18</td>
</tr>
<tr>
<td>6</td>
<td>Xai</td>
<td>90</td>
<td>2/6</td>
<td>91 ± 21</td>
</tr>
<tr>
<td>8</td>
<td>X</td>
<td>300</td>
<td>0/8</td>
<td>74 ± 13</td>
</tr>
<tr>
<td>8</td>
<td>Heparin</td>
<td>200 U/Kg/min IV</td>
<td>8/8</td>
<td>&gt;180*</td>
</tr>
</tbody>
</table>

*P < .001 compared with saline controls or factor X infusion.
† The indicated times refer to the interval required for total occlusion in 3 of 8, 4 of 7, and 4 of 6 animals receiving factor Xai at doses of 150, 120, and 90 µg/kg, respectively. The other animals in these groups did not occlude in more than 180 minutes.
‡ P < .05 compared with saline controls of factor X infusion.

The indicated number of animals (N) received a single intravenous bolus of either saline alone (8 mL), or factor X or Xai in saline (volume, approximately 8 mL), or heparin (5,000 U bolus followed by the given rate of infusion) via a peripheral vein in an extremity. The number of animals that remained patent is shown (numerator) divided by the total number of animals in that group (denominator).

Changes in hemostatic parameters. The effect of Xai infusions on systemic hemostatic parameters was studied on blood samples from treated and control animals. Plasma samples obtained 1 hour after the bolus of factor Xai (300 µg/kg) demonstrated a clear prolongation of the PT, but there was no significant change in the PT in animals receiving factor X (300 µg/kg) (Fig 5A). The time course of changes in the PT showed the greatest prolongations up to 20 to 40 minutes after the infusion, with a return of the clotting time to the baseline by 180 minutes later (data not shown), consistent
with the clearance of bovine Xai at this time. Tests of platelet function, including aggregation in response to collagen, ADP, and U46619, were not altered in animals receiving factor Xai (data not shown).

To assess the effect of factor Xai on extravascular hemostasis, blood loss after a standardized abdominal incision was determined (Fig 5B). Animals infused with saline or factor X did not bleed excessively compared with untreated controls. In contrast, there was a striking increase in blood loss into the wound at doses of factor Xai corresponding to 120, 150, and 300 μg/kg. When the abdominal wound model was performed in factor Xai-treated animals that received fibrinogen, radioactivity in the gauze that had been placed in the wound was consistently (minimum of 5- to 6-fold) greater than that of controls. Excessive bleeding into the abdominal incision was also observed at a heparin dose of 200 μg/kg/min (this dose is close to the threshold dose for prevention of intravascular thrombus formation in this model). These data were paralleled by clinical observations that excessive bleeding occurred throughout the experimental procedures, eg, at sites of manipulation, incisions, and catheter insertions.

Discussion

The results of this study indicate that active site-blocked factor Xa inhibits thrombosis, in an electrically induced coronary thrombosis model, and prevents extravascular hemostasis, in response to a standardized abdominal incision, at comparable concentrations. In view of the central role of factor Xa in the prothrombinase complex, the major pathway leading to thrombin formation, it is to be expected that factor Xai-mediated inhibition of Xa assembly into the activation complex would seriously impair the procoagulant mechanism. Factor Xai may also be exerting its effect, in part, by substituting for the substrate, factor X. In view of the virtually complete clearance of infused factor Xai by 180 minutes, whereas the injured vessel remains patent at this
Fig 2. Representative transmission electron micrographs of thrombi from the left circumflex artery segments of animals infused with factor Xai (A through C) or saline (controls) (D through F). (A) and (D) are lower power micrographs (original magnification X7,500) showing the presence of cellular and fibrinous elements in both thrombi. At higher magnification (X25,000), (B) and (E) show the periodicity of the fibrin present in both the thrombi. Similarly, high power magnification (X20,000) of the cellular elements in both the thrombi (C) and (F) show the presence of mostly degranulated platelets.
time, it appears that the essential period in which factor Xai antithrombotic activity is required to prevent occlusive thrombosis is during the early time (up to about 2 hours) after electrically induced vessel wall injury. To evaluate effective plasma levels of Xai in detail, it will be necessary to compare results with bovine Xai (used in our studies) with canine Xai, to account for possible differences due to species specificity.

The parallel perturbation of intravascular and extravascular clotting by factor Xai contrasts with our previous study with factor IXai; IXai inhibited only thrombosis without affecting extravascular bleeding. This leads us to propose the following tentative outline for the procoagulant mechanisms for thrombosis/extravascular hemostasis in this canine model (Fig 6). Electrically induced vessel injury has been speculated to occur through the generation of oxygen free radicals, resulting in increased vascular permeability and thereby exposing subendothelial elements, both basement membrane components and cells that express tissue factor, to the blood. Because factor Xa formed by factor IXa-VIIIa assembles into the prothrombinase complex, Xai functions as an inhibitor at this point as well.

The situation in the extravascular space, as demonstrated by bleeding in response to the abdominal incision, is different.
FACTOR XAI ON CORONARY THROMBOSIS

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Fig 5. Effect of factor Xai infusion on the PT of canine plasma (A) and on bleeding in response to a standardized abdominal incision (B). (A) The PT was performed on citrated plasma samples from animals infused with either saline (control; N = 36), factor X (300 pg/kg; N = 8), factor Xai at the indicated concentration (N = 8, 7, and 6 at Xai concentrations of 150, 120, and 90 pg/kg, respectively), or heparin (5,000 U bolus followed by 200 U/kg/min; N = 8). The clotting times of the control group (a), and of the experimental groups before factor X or Xai infusion (m) are shown. (B) The amount of blood loss into a standardized abdominal incision (1 X 5 cm) was measured by placing a 4 X 4 inch preweighed gauze into the incision for 5 minutes, removing, and weighing the gauze to determine accumulated blood. The experimental groups were the same as in (A), and blood loss before the indicated treatment (m) and 1 hour later (a) is shown. (B) Blood loss in saline-infused controls. In (A) and (B), the mean ± SD is shown. *P < .05, **P < .02, and ***P < .001 compared with controls.

The hemostatic response remains intact in the presence of factor IXai, indicating that IXa-independent mechanisms predominate in initiation of coagulation in the subcutaneous tissues. This is consistent with the observations of Weiss and Lages, in which factor VIIa-induced activation of factor X was the major contributor to factor Xa formation in bleeding wounds. Predominance of factor VIIa-mediated activation of factor X might have been expected in the skin in view of the abundance of tissue factor in subcutaneous tissues, in concert with previous observations that higher levels of tissue factor favor direct factor X activation. Because factor Xa formed by the action of factor VIIa interacting with subcutaneous tissue factor must enter the prothrombinase complex to effectively promote prothrombin activation, Xai is an inhibitor of clotting in the extravascular space. An alternative, although less likely, mechanism of Xai action would involve nonspecific binding of the gamma-carboxylated, active site-blocked enzyme to acidic phospholipid surfaces, thereby preventing interaction with native coagulation proteins.

Based on the results of our experiments with Xai, one would anticipate that inhibition of thrombin or Xa, procoagulant enzymes in the final common pathway, by any means would lead to a bleeding diathesis. Although this would appear to conflict with recent studies that thrombin and factor Xa inhibitors can be administered without adverse bleeding consequences, there are likely to be explanations for this potential discrepancy. On the one hand, the inhibitors used may not be as effective as Xai, and thus allow small amounts of active enzymes to escape inhibition and to initiate the procoagulant hemostatic response. Alternatively, the means used to test the bleeding tendency in the studies with factor Xa and thrombin inhibitors may not have been a significant enough stress to the hemostatic system to manifest the bleeding tendency. This emphasizes the importance of developing additional standardized methods for measuring the hemostatic response to stress to facilitate comparison of studies using antithrombotic agents.

Although the relevance of electrically induced thrombosis to naturally occurring pathologic clotting in a coronary artery is not clear, our results with IXai and Xai suggest the potential utility of this model for dissecting the contributions of different mechanisms for activation of coagulation (ie, IXa/VIIa-dependent and -independent mechanisms) in vivo from the obligatory final common pathway leading to thrombin formation. If the electrical model can be extrapolated to human thrombotic disease, it is possible that inhibition of factor IXa assembly into the intrinsic factor X activation complex may provide a selective means to exploit the differences in thrombosis and extravascular hemostasis.

Fig 6. Schematic depiction of the procoagulant mechanism activated by an electrically induced vessel injury, and a mechanically induced, subcutaneous wound. IXai, active-site blocked factor IXa; Xai, active-site blocked factor Xa.
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REFERENCES


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