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Active Site-blocked Factor IXa Prevents Intravascular Thrombus Formation in the Coronary Vasculature without Inhibiting Extravascular Coagulation in a Canine Thrombosis Model

Claude R. Benedict,* Jane Ryan,‡ Barry Woltzky,§ Robert Ramos,∥ Marlene Gerlach,* Pim Tijburg,¶ and David Stern∥

*Department of Internal Medicine and Division of Cardiology, University of Texas Medical School at Houston, Houston, Texas 77225; ‡Department of Physiology and Cellular Biophysics, Columbia University, College of Physicians and Surgeons, New York 10032; and ∥Department of Molecular Genetics, Hoffmann-LaRoche, Inc., Nutley, New Jersey 07115

Abstract

To assess the contribution of Factor IX/IXa, to intravascular thrombosis, a canine coronary thrombosis model was studied. Thrombus formation was initiated by applying current to a needle in the 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-glycyl-arginyl-Factor IXa (IXa), a competitive inhibitor of Factor IXa assembly into the intrinsic Factor X activation complex, bovine Factor IX, or heparin. Animals receiving saline or Factor IX developed coronary occlusion due to a fibrin/platelet thrombus in 70±11 min. In contrast, infusion of IXa prevented thrombus formation completely (>180 min) at doses of 460 and 300 μg/kg, and partially blocked thrombus formation at 150 μg/kg. IXa attenuated the accumulation of 125I-fibrinogen/fibrin at the site of the thrombus by ~67% (P < 0.001) and resulted in ~26% decrease in serotonin release from platelets in coronary sinus (P < 0.05). Hemostatic variables in animals receiving IXa, remained within normal limits. Animals given heparin in a concentration sufficient to prevent occlusive thrombosis had markedly increased bleeding, whereas heparin levels that maintained extravascular hemostasis did not prevent intracoronary thrombosis. This suggests that Factor IX/IXa can contribute to thrombus formation, and that inhibition of IXa participation in the clotting mechanism blocks intravascular thrombosis without impairing extravascular hemostasis. (J. Clin. Invest. 1991. 88:1760–1765.) Key words: hemostasis • heparin • cloting mechanisms • coronary thrombosis • fibrin

Introduction

In addition to its role in normal hemostasis, several pieces of evidence suggest that IX/IXa may contribute to thrombosis. Infusion of Factor IXa has been shown to result in the forma-

tion of thrombi (1), and Factor IXa is a potent thrombogenic agent in the Wessler stasis thrombosis model (2). Since Factor IXa participates in procoagulant reactions as part of the Factor IXa-VIIa-X activation complex, the availability of active site-blocked IXa (glutamyl-glycyl-arginyl-Factor IXa or IXai),1 which in vitro prevents IXa assembly into the complex, allows for the design of studies to assess the role of this procoagulant enzyme in vivo (3–7). These considerations led us to examine the effect of IXai in an experimental model of coronary thrombosis. The results indicate that intravenous infusion of bovine Factor IXa into dogs at doses to achieve a plasma level of 1–2 μg/ml blocks coronary thrombosis without impairing the hemostatic response to injuries at extravascular sites (a standardized abdominal incision, incision sites in the chest wall, and catheter insertion sites). These data suggest that Factor IX/IXa may have an important role in thrombosis, and that intervention in the procoagulant pathway at this level may selectively block intravascular thrombosis.

Methods

Preparation of Factor IX and Factor IXai. Bovine Factor IX was purified to homogeneity by previously described methods (5, 8), and activated during a 30-min incubation at 37°C with Factor Xa (Enzyme Research Laboratories, Inc., South Bend, IN) (20%; wt/wt) in the presence of CaCl2 (10 mM), as described previously (5, 9). The reaction was stopped by the addition of EDTA (10 mM), and chromatographed on FPLC Mono Q (Pharmacia Inc., Piscataway, NJ). Factor IXa eluted as the column was developed with a linear salt gradient (0.1–1 M NaCl), whereas Factor Xa did not adhered to the column. The final product contained 90–95% Factor IXa with the remainder comprised by residual zymogen. Factor IXa was inactivated by incubation with glutamyl-glycyl-arginyl-chloromethylketone (Calbiochem-Behring Corp., La Jolla, CA) (4), using ~50-fold M excess of inhibitor to Factor IXa for 10 min at 37°C, and then dialyzed exhaustively versus Tris-buffered saline (Tris, 20 mM, pH 7.4; NaCl, 100 mM). Glutamyl-glycyl-arginyl-Factor IXa was termed active site–blocked Factor IXa or IXai, and had no residual procoagulant activity. Factor IXa was also dialyzed extensively versus Tris-buffered saline before use in infusion studies. Furthermore, incubation of IXai with thrombin did not lead to significant loss of enzyme activity, suggesting that all free inhibitor was removed during the dialysis step. Electric current model of coronary thrombosis and infusion studies. In vivo coronary thrombosis was induced with electric current, as described previously (10). In brief, the left circumflex coronary artery was

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1. Abbreviations used in this paper: APTT, activated partial thromboplastin time; IXai, glutamyl-glycyl-arginyl Factor IXa or active site–blocked Factor IXa.

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isolated and instrumented with a Doppler flow probe to measure coronary blood flow velocity, a needle electrode placed intraluminally to initiate thrombus formation, and sampling catheters in the left atrium and coronary sinus. Length-segment crystals were placed in the anterior and posterior myocardial wall to measure alterations in myocardial contractility with the onset of ischemia due to total occlusion. After instrumentation and a 30-min period to allow stabilization of hemodynamic variables, current was applied (150 μA) to the needle electrode until a 50% increase in blood flow velocity occurred (this correlates with a 40–50% decrease in cross-sectional area of the lumen due to thrombus formation at the site of placement of the electrode [10]). After cessation of the current, animals received an intravenous infusion of either saline (8 ml), Factor IXa (at the indicated concentration in a volume of ~8 ml of saline), Factor IX (460 μg/kg in a volume of ~8 ml of saline), or heparin (5,000 U i.v. as bolus followed by 100 or 200 U/kg per min). Where indicated, animals also received 125I-fibrinogen (5 μCi in 1 ml i.v. of saline) at the time the current was discontinued. Fibrinogen was purified and radioiodinated as described previously (specific radioactivity of 130 μCi/mg) [11, 12].

Determination of 125I-fibrinogen/fibrin accumulation in coronary artery segments and assessment of serotonin release. In animals infused with 125I-fibrinogen either at the time of vessel occlusion or 180 min 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. Accumulation of radioactivity in the circumflex artery was expressed as a ratio of that measured in a segment of similar length and weight from the undisturbed left anterior descending coronary artery. Serotonin levels in EDTA-anticoagulated platelet-poor plasma samples (coronary sinus minus left atrial), were determined by a radioenzymatic assay [13] every 20 min as an index of in vivo platelet aggregation (10) throughout the experiment.

Assessment of coagulation, bleeding parameters, and Factor IXa levels in canine plasma. The activated partial thromboplastin time (APTT) and platelet aggregation studies in response to ADP, collagen, and U46619 (a stable thromboxane A2 analogue; Upjohn, Kalamazoo, MI) were performed on samples of citrated blood (0.4%) from saline-, Factor IX-, or IXa-infused animals by standard methods. The bleeding tendency at extravascular sites was assessed using modified incisional bleeding time: a uniform 1-cm deep, 5-cm-long abdominal wall incision was made, and a preweighed 4 × 4-in. gauze was inserted for 5 min. The gauze was then removed, reweighed, and the weight of blood loss quantitated. The level of Factor IX/IXa antigen in dog plasma was determined by a radioimmunoassay using goat antibody prepared to bovine Factor IX as described previously [14]. This antibody does not distinguish between bovine Factor IX and IXa [14], and did not detect dog Factor IX antigen at a 1:1 dilution of dog plasma. Dog plasma samples or bovine Factor IX diluted in control dog plasma were incubated overnight with 125I-bovine Factor IX (radioiodinated as described previously [5]) and affinity purified goat anti-bovine Factor IX antibody (500 ng/ml). Bound 125I-bovine Factor IX was precipitated by addition of protein G-Sepharose. The limit of sensitivity in this assay was 100 ng/ml, which corresponded to 80% binding on the standard curve.

Statistical analysis. Data were analyzed by one-way analysis of variance. In each figure and Table I, mean values ±SD are shown.

Results

Time to coronary occlusion. Application of electric current to the left circumflex coronary artery for 62±21 min resulted in a 40–50% decrease in cross-sectional area of the lumen due to thrombus formation as observed previously [10]. After discontinuation of the current, complete cessation of blood flow occurred within 70±31 min (Fig. 1 top and Table I), due to continuing thrombus formation, eventually leading to an occlusive fibrin/platelet thrombus [10], which on histologic examination resembled thrombi observed in coronary vessels from patients [10, 15, 16]. If the animal received a single intravenous bolus of Factor IXa (300 μg/kg) at the time the current was discontinued, although coronary blood flow initially appeared to decrease, it never ceased and subsequently it returned to levels observed before discontinuing the current (Fig. 1 bottom). Examination of the vessel from animals infused with Factor IXa revealed only a small, nonocclusive thrombus containing fibrin and platelets (data not shown), compared with occlusive thrombi of the same apparent histologic composition in saline-treated controls. Hemodynamic variables (arterial blood pressure, heart rate, anterior and posterior wall myocardial contractility, and phasic and mean coronary blood flow) were not affected by the infusion of Factor IXa, and, since the vessel remained patent, posterior wall contractility was maintained (Fig. 1 bottom). A relatively selective antigen assay for bovine Factor IX/IXa demonstrated a rise in antigen level to ~2 μg/ml by 20 min after the intravenous infusion of IXa, and a gradual decline to ~1 μg/ml over the next 100 min (Fig. 2).

The inhibitory effect of IXa on thrombus formation was dose dependent, being effective in all animals receiving 460 μg/kg i.v. or i.c., and 300 μg/kg i.v., but in only 50% of animals receiving 150 μg/kg i.v. (Table I). In contrast, intravenous infusion of the same levels of the zymogen, Factor IX, did not prolong the time to reocclusion beyond that observed in saline-treated controls (Table I). When animals were anticoagulated by the administration of heparin (5,000 U i.v. as bolus followed by 200 U/kg per min), the left circumflex coronary artery also remained patent (Table I), although lower levels of heparin (5,000 U i.v. as an bolus followed by 100 U/kg per min; n = 5) did not prevent coronary occlusion (Table I).

Radiolabeled fibrinogen accumulation in thrombus. Since IXa would be expected to interfere primarily with the procoagulant mechanism ultimately leading to the generation of fibrin, it was important to assess its effect on the deposition of radiolabeled fibrinogen/fibrin in the thrombosed coronary segment (Fig. 3 A). Animals were infused with 125I-fibrinogen at the time the current was discontinued, and a 125I-fibrinogen/fibrin accumulation ratio (the ratio of radioactivity deposited per milligram of tissue in the circumflex artery at the site of placement of the needle electrode (thrombosed site), or proximal or distal to this area, compared with radioactivity deposited in the non-manipulated left anterior descending coronary artery) was measured. In the presence of IXa, 125I-fibrinogen/fibrin accumulation ratios decreased in the thrombosed and distal segments by ~26% reduced in animals infused with IXa compared with controls (Fig. 3 B). Similarly, serotonin levels in the thrombus, which correlate closely with the number of platelets in the thrombus based on previous studies with 111I-indium–labeled platelets [17], were decreased by ~20% (data not shown).

Alterations in hemostatic variables. To determine the effect of the IXa infusions on systemic hemostatic variables, a range of assays was performed on blood samples from treated and control animals. Plasma from animals infused with IXa or IX
did not show significant lengthening of the APTT, although studies on animals receiving heparin, at levels which prevented coronary occlusion, demonstrated prolongation of the APTT (Fig. 4A). Tests of platelet function, including aggregation in response to collagen, ADP, and U46619, were not altered in animals receiving IXai (data not shown).

The effect of IXai on clotting in response to a cutaneous abdominal wound was assessed after a standardized incision by weighing the amount of blood adsorbed by a sponge placed in the wound over 5 min (Fig. 4B). Animals infused with either saline, Factor IX, or IXai did not bleed excessively compared with untreated controls. Consistent with this observation, when the abdominal wound model was performed on IXai-treated animals infused with $^{125}$I-fibrinogen, radioactivity that accumulated in the gauze reached the same levels as that observed in saline controls, suggesting comparable deposition of $^{125}$I-fibrinogen/fibrin. In contrast, animals receiving heparin at levels required to prevent occlusive coronary thrombosis (200 U/kg per min) had markedly increased bleeding. The dose of heparin used was then steadily decreased until a level was reached at which there was only moderately increased bleeding from the abdominal wound (100 U/kg per min) (Fig. 4B). At this concentration of heparin, however, occlusion of the left circumflex coronary artery occurred at similar time intervals as noted in saline-treated control animals (Table I). The contrast between the increased bleeding tendency of animals receiving heparin (200 U/kg per min, the amount of heparin required to prevent formation of an occlusive thrombus) and the apparent lack of bleeding in animals treated with IXai was qualitatively evident throughout the experimental manipulations, as regards blood loss at incisions in the chest wall and catheter insertion sites.
Table I. Effect of Factor IX and IXai on Coronary Occlusion in the Electric Current-Induced Coronary Thrombosis Model

<table>
<thead>
<tr>
<th>n</th>
<th>Agent</th>
<th>Dose</th>
<th>Occlusion</th>
<th>Time to occlusion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>Saline</td>
<td>—</td>
<td>36/36</td>
<td>70±11</td>
</tr>
<tr>
<td>3</td>
<td>IXai</td>
<td>460 μg/kg i.c.</td>
<td>0/3</td>
<td>&gt;180*</td>
</tr>
<tr>
<td>5</td>
<td>IXai</td>
<td>460 μg/kg i.v.</td>
<td>0/5</td>
<td>&gt;180*</td>
</tr>
<tr>
<td>5</td>
<td>IXai</td>
<td>300 μg/kg i.v.</td>
<td>0/5</td>
<td>&gt;180*</td>
</tr>
<tr>
<td>6</td>
<td>IXai</td>
<td>150 μg/kg i.v.</td>
<td>3/6</td>
<td>102±31†</td>
</tr>
<tr>
<td>5</td>
<td>IX</td>
<td>460 μg/kg i.v.</td>
<td>5/5</td>
<td>79±13</td>
</tr>
<tr>
<td>8</td>
<td>Heparin</td>
<td>200 U/kg per min i.v.</td>
<td>0/8</td>
<td>&gt;180*</td>
</tr>
<tr>
<td>5</td>
<td>Heparin</td>
<td>100 U/kg per min i.v.</td>
<td>5/5</td>
<td>58±14</td>
</tr>
</tbody>
</table>

The indicated number of animals (n) were infused with either saline alone (8 ml), Factor IX, or IXai in saline (volume: ~8 ml), or heparin (5,000 U bolus followed by the indicated rate of infusion). The number of animals that developed coronary artery occlusion (complete cessation of flow) is shown divided by the total number of animals in that group. The time required for total coronary occlusion to develop is also shown. i.c.: infusion directly into the left circumflex artery just proximal to the needle electrode; i.v.: infusion via a peripheral vein in an extremity.

* P < 0.001 compared controls infused with saline or Factor IX.
† Time required for occlusion in three of the animals in this group. The other three animals did not develop coronary artery occlusion in >180 min.

Discussion

In view of the paucity of tissue factor in the intravascular space (18, 19), as well as the presence in plasma of an inhibitor of the tissue factor pathway synthesized by endothelium, lipid-associated coagulation inhibitor, or extrinsic pathway inhibitor (20–22), it seemed logical that the intrinsic system could play an important role in intravascular clot formation. In support of this, both endothelium and platelets have been shown to bind Factor IX/IXa specifically, and to promote assembly of the intrinsic Factor X activation complex (5–7). Furthermore, preliminary studies in which tumor-bearing mice infused with tumor necrosis factor/cachectin developed intravascular clot for-
mation localized to the tumor bed, showed that thrombosis could be blocked by the infusion of active site-blocked Factor IXa (data not shown).

Using a canine coronary thrombosis model, our results indicate that a single, peripherally administered IV bolus of IXa blocked thrombus formation in a dose-dependent manner, as judged by maintenance of blood flow and inhibition of fibrinogen/fibrin accumulation. Inhibition of intravascular thrombus formation in the presence of IXa was not accompanied by excessive bleeding; i.e., the same concentrations of IXa that were effective in preventing coronary occlusion did not lead to increased bleeding at the site of a standardized abdominal incision, or at intravenous and chest wall incision sites. A possible explanation for this is that the contribution of Factor IXa to Factor X activation may be less important in the extravascular space under our experimental conditions as compared with direct tissue factor-Factor VIIa-activated clotting activity of Factor X. This hypothesis is supported by the work of Weiss and Lages (23), indicating the central role of Factor VIIa-mediated activation of Factor X in blood obtained from bleeding wounds. In this context, previous work has shown that in the presence of high concentrations of tissue factor, as would be true in the extravascular space, especially in skin (24), Factor VIIa/tissue factor–mediated activation of Factor X is favored compared with activation of Factor IX (25), whereas with limited amounts of tissue factor, activation of Factor IX is enhanced in vitro (26, 27) and in vivo (28). Another explanation involves the possibility that activation of endogenous Factor IX may occur to a much greater extent at the site of the extravascular wound, and IXa may not be present at high enough concentrations to completely block its assembly into the intrinsic Factor X activation complex. It would be expected that if Factor IXa participation in clotting was blocked completely, the type of bleeding disorder observed in severe hemophilia B would occur (3). In contrast to the effect of IXa, the dose of heparin that prevented intracoronary arterial occlusion in this model also resulted in excessive bleeding at the site of a wound, whereas lower levels of heparin, which normalized the bleeding tendency, failed to prevent coronary thrombosis.

Although the precise mechanism through which IXa exerts its effect on intravascular versus extravascular coagulation remains to be defined, it is clear that interference with the procoagulant mechanism at the level of Factor IX/IXa can prevent experimentally induced thrombosis within the vessel lumen in the absence of excessive bleeding in the tissue. These observations lend support to a potentially important role for Factor IX/IXa in thrombosis, and suggest that inhibition of IXa can have a potentially selective effect on the coagulation mechanism.

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