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

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## 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 (IXai), 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 IXai prevented thrombus formation completely (> 180 min) at doses of 460 and 300 μg/kg, and partially blocked thrombus formation at 150 μg/kg. IXai attenuated the accumulation of $^{125}$I-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 IXai, 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 • clotting 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 formation 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-VIIIa-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 IXai 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 XIa (Enzyme Research Laboratories, Inc., South Bend, IN) (20%; wt/wt) in the presence of $CaCl_2$ (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 XIa did not adhere 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 30 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 IX 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.

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 IXai (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 $^{125}$I-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 $^{125}$I-fibrinogen/fibrin accumulation in coronary artery segments and assessment of serotonin release.* In animals infused with $^{125}$I-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 IXai levels in canine plasma.* The activated partial thromboplastin time (APTT) and platelet aggregation studies in response to ADP, collagen, and U46619 (a stable thromboxane $A_2$ analogue; Upjohn, Kalamazoo, MI) were performed on samples of citrated blood (0.4%) from saline-, Factor IX-, or IXai-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/IXai 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 IXai (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 $^{125}$I-bovine Factor IX (radioiodinated as described previously [5]) and affinity purified goat anti–bovine Factor IX antibody (500 ng/ml). Bound $^{125}$I-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 a 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±11 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 IXai (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 IXai 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 IXai, and, since the vessel remained patent, posterior wall contractility was maintained (Fig. 1 *bottom*). A relatively selective antigen assay for bovine Factor IX/IXai demonstrated a rise in antigen level to ~ 2 μg/ml by 20 min after the intravenous infusion of IXai, and a gradual decline to ~ 1 μg/ml over the next 100 min (Fig. 2).

The inhibitory effect of IXai 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 IXai 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 $^{125}$I-fibrinogen at the time the current was discontinued, and a $^{125}$I-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 nonmanipulated left anterior descending coronary artery) was measured. In the presence of IXai, $^{125}$I-fibrinogen/fibrin accumulation ratios decreased in the thrombosed and distal segments by ~ 67 and 61%, respectively, whereas there was no difference in the segment proximal to the lesion. Serotonin release in the coronary sinus measured to provide an estimate of platelet activation/release in the circumflex artery was modestly (~ 26%) reduced in animals infused with IXai 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 $^{111}$I-indium–labeled platelets (17), were decreased by ~ 20% (data not shown).

*Alterations in hemostatic variables.* To determine the effect of the IXai infusions on systemic hemostatic variables, a range of assays was performed on blood samples from treated and control animals. Plasma from animals infused with IXai or IX

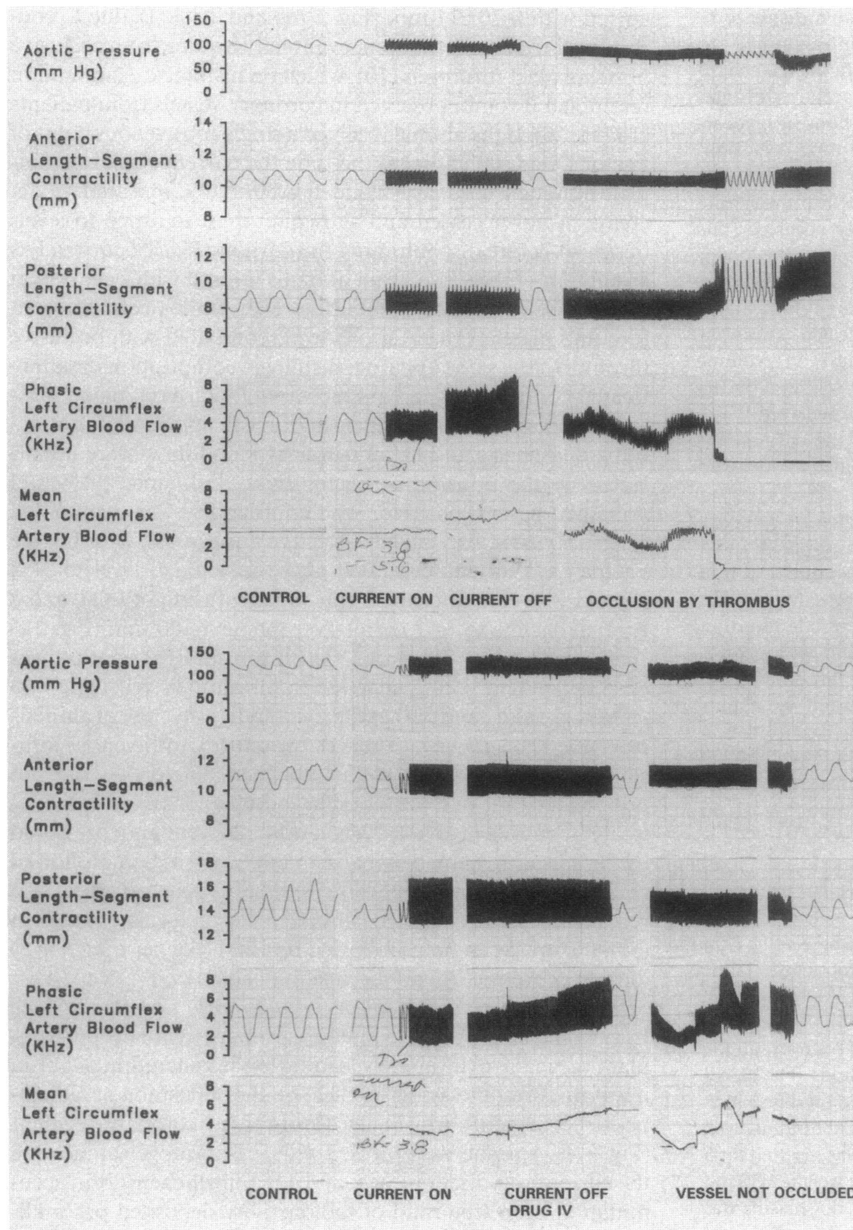


*Figure 1.* Effect of Factor IXai on coronary thrombosis in a canine model. (*Top*) control: base line measurement of aortic pressure, anterior and posterior myocardial wall contractility, and phasic and mean circumflex arterial blood flow; current on: after instrumentation of the left circumflex artery, current was applied to the needle electrode; current off: current was continued until a 50% increase in phasic and mean blood flow velocity (corresponding to a 40–50% decrease in cross-sectional luminal area) occurred. At this time the current was stopped and saline was given; occlusion by thrombus: 74 min after giving saline the vessel was totally occluded (note the absence of circumflex blood flow and decrease in the posterior wall contractility due to ischemia of the myocardium supplied by the occluded artery). (*Bottom*) control, current on and current off/drug intravenous depict the same variables in an animal infused with Factor IXai (300 μg/kg). Factor IXai was given intravenous when the current was stopped. The vessel did not occlude throughout the study which was terminated at 180 min.

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. 4 *A*). 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. 4 *B*). 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. 4 *B*). 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*

| n | Agent | Dose | Occlusion | Time to occlusion (mean±SD) |
|---|---|---|---|---|
| | | | | *min* |
| 36 | Saline | — | 36/36 | 70±11 |
| 3 | IXai | 460 μg/kg i.c. | 0/3 | >180* |
| 5 | IXai | 460 μg/kg i.v. | 0/5 | >180* |
| 5 | IXai | 300 μg/kg i.v. | 0/5 | >180* |
| 6 | IXai | 150 μg/kg i.v. | 3/6 | 102±31‡ |
| 5 | IX | 460 μg/kg i.v. | 5/5 | 79±13 |
| 8 | Heparin | 200 U/kg per min i.v. | 0/8 | >180* |
| 5 | Heparin | 100 U/kg per min i.v. | 5/5 | 58±14 |

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-

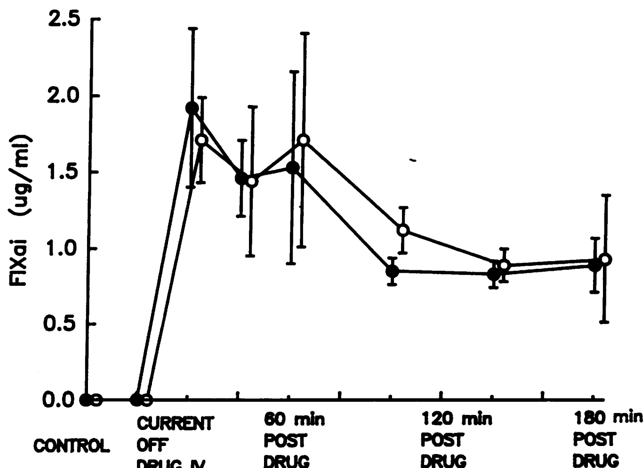


*Figure 2.* Level of bovine Factor IX/IXai antigen in dog plasma using a species-specific radioimmunoassay. Note the increase in bovine Factor IX/IXai antigen after administration of 300 μg/kg i.v. of Factor IXai (*n* = 4). (*Open circles*) left atrial plasma sample; (*Closed circle*) coronary sinus plasma sample. The error bars are standard deviations of the mean.

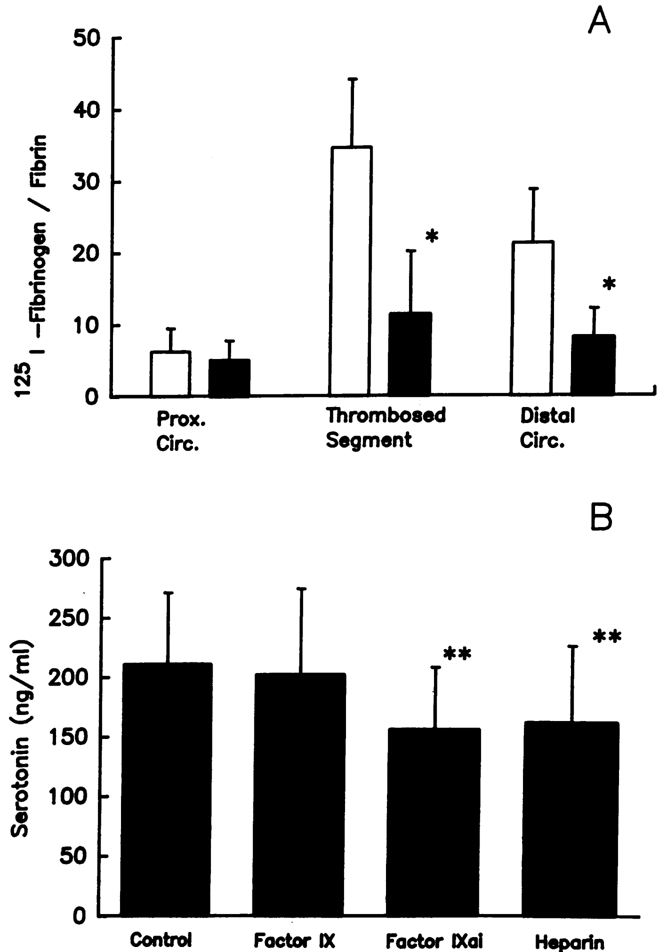


*Figure 3.* Effect of Factor IXai infusion on the deposition of $^{125}$I-fibrinogen/fibrin in the left circumflex artery (*A*) and on serotonin levels in the coronary sinus (*B*). (*A*) Animals were subjected to electrical stimulation of the left circumflex coronary artery, which was discontinued when a 50% increase in blood flow velocity was observed. At this time, $^{125}$I-fibrinogen (5 μCi) was infused alone (*n* = 4; *open bar*) or in the presence of IXai (300 μg/kg; *n* = 4; *closed bar*). In animals infused with $^{125}$I-fibrinogen alone, at the time of total occlusion (70±11 min), the animal was killed and the left anterior descending coronary artery and the left circumflex coronary artery were excised. In animals receiving IXai, the coronary artery segments were obtained after death at 180 min. Circumflex and left anterior descending coronary artery segments were obtained as described in the text, and the $^{125}$I-fibrinogen/fibrin-accumulation ratio was determined in the proximal, thrombosed, and distal portions of the left circumflex artery. (*B*) Serotonin release into coronary sinus blood was determined in animals infused with either saline (*control*; *n* = 36), Factor IX (460 μg/kg; *n* = 5), Factor IXai (300 μg/kg; *n* = 5), or heparin (200 U/kg per min; *n* = 8). Serotonin levels (coronary sinus minus left atrial levels) were measured every 20 min from the time the current was discontinued, and the values shown reflect the peak levels (observed at 60 min after stopping the current). In both *A* and *B*, the mean±SD is shown in each case, and *$P < 0.02$ and **$P < 0.05$.

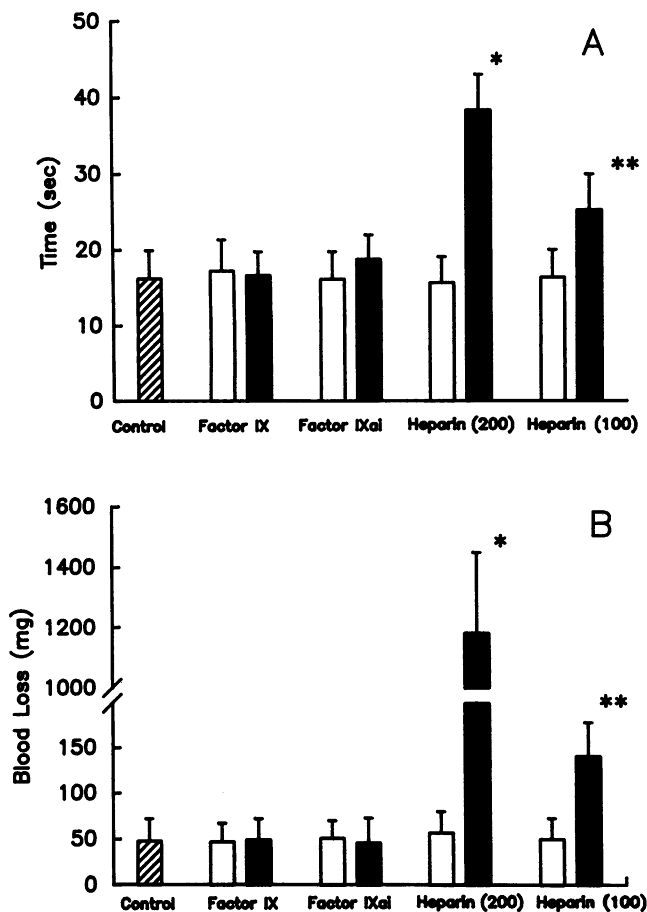


*Figure 4.* Effect of Factor IXai infusion on the APTT of dog plasma (*A*) and on bleeding in response to a standardized abdominal incision (*B*). (*A*) The APTT was performed on citrated plasma samples from animals infused with either saline (*control*; $n = 36$), Factor IX (460 μg/kg; $n = 5$), Factor IXai (460 μg/kg; $n = 5$), heparin 200 (200 U/kg per min; $n = 8$), or heparin 100 (100 U/kg per min; $n = 5$). The clotting times of the control group (*striped bar*), the experimental groups before the infusion (*open bar*), and 1 h after the infusion (*closed bar*) are shown. (*B*) The amount of blood loss into a standardized abdominal incision (1 × 5 cm) was measured by placing a 4 × 4-in preweighed gauze into the incision for 5 min, and 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 (*open bars*) and 1 h later (*closed bars*) are shown. The striped bars show blood loss in saline-infused controls. In both *A* and *B*, the mean±SD is shown in each case. $*P < 0.001$ and $**P < 0.05$.

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 IXai 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 IXai was not accompanied by excessive bleeding; i.e., the same concentrations of IXai 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 VII/VIIa–mediated activation 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 IXai 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 IXai, 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 IXai 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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