Heparin-Stimulated Inhibition of Factor IXa Generation and Factor IXa Neutralization in Plasma

By Jean Pieters, Theo Lindhout, and George Willems

Generation and inhibition of activated factor IXa was studied in factor Xla-activated plasma containing 4 mmol/L free calcium ions and 20 µmol/L phospholipid (25 mol% phosphatidylserine/75 mol% phosphatidylcholine). Interference of other (activated) clotting factors with the factor Xla activity measurements could be avoided by using a highly specific and sensitive bioasay. Factor IXa generation curves were analyzed according to a model that assumed Michaelis-Menten kinetics of factor Xla-catalyzed factor IXa formation and pseudo first order kinetics of inhibition of factor Xla and factor IXa. In the absence of heparin, factor IXa activity in plasma reached final levels that were found to increase with increasing amounts of factor Xla used to activate the plasma. When the model was fitted to this set of factor IXa generation curves, the analysis yielded a rate constant of inhibition of factor Xla of 0.7 ± 0.1 min⁻¹ and a k_cat/K_m ratio of 0.29 ± 0.01 (nmol/L)⁻¹ min⁻¹. No neutralization of factor IXa activity was observed (the estimated rate constant of inhibition of factor IXa was 0). Thus, in the absence of heparin, the final level of factor IXa in plasma is only dependent on the initial factor Xla concentration. While neutralization of in situ generated factor IXa in normal plasma was negligible, unfractionated heparin dramatically enhanced the rate of inactivation of factor IXa (apparent second order rate constant of inhibition of 5.2 min⁻¹ per µg heparin/mL). The synthetic pentasaccharide heparin, the smallest heparin chain capable of binding antithrombin III, stimulated the inhibition of in situ generated factor IXa, but sevenfold less than unfractionated heparin (k = 0.76 min⁻¹ per µg pentasaccharide/mL). We found that free calcium ions were absolutely required to observe an unfractionated heparin and pentasaccharide-stimulated neutralization of factor IXa activity. Factor Xla inhibition (pseudo first order rate constant of 0.7 min⁻¹) was not affected by unfractionated heparin or pentasaccharide in the range of heparin concentrations studied.

© 1990 by The American Society of Hematology.

Materials and Methods

The synthetic peptide substrate Bz-Ile-Glu-(piperidy1)-Gly-Arg-p-nitro-anilide hydrochloride (S-2337) was purchased from AB Kabi Diagnostica (Stockholm, Sweden). Factor IX deficient plasma was purchased from DiaMed AG (Morat, Switzerland). Ovalbumin was a product of Sigma Chemical Co (St Louis, MO). All reagents used were of the highest grade commercially available. The Fourth International Standard for Heparin (ISH; 180 USP U/mg) was obtained from the National Institute for Biological Standards and Control, London, UK. The synthetic pentasaccharide heparin (800

From the Department of Biochemistry and Research Institute of Cardiovascular Diseases, University of Limburg, Maastricht, The Netherlands.

Submitted November 22, 1989; accepted April 5, 1990.

Address reprint requests to Theo Lindhout, PhD, Department of Biochemistry, University of Limburg, PO Box 616, 6200 MD Maastricht, The Netherlands.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1990 by The American Society of Hematology.

0006-4971/90/7603-023$3.00/0

Blood, Vol 76, No 3 (August 1), 1990: pp 549-554
anti-factor Xa IU/mg) was a kind gift from Institut Choay, Paris, France.

**Proteins.** Human factor IX, human factor XI, and factor Xa were purified and quantitated as described earlier. All bovine clotting factors were prepared and quantitated as previously described. Recombinant desulfato-hirudin variant 1 (CGP39393) was kindly provided by Dr. R.B. Wallis (Ciba-Geigy, Horsham, UK).

**Phospholipid vesicles.** Phospholipid vesicles were made from a mixture of phosphatidylserine (PS) and phosphatidylycholine (PC) as previously described. Vesicles composed of 25 mol% PS and 75 mol% PC were used throughout this study.

**Preparation of human normal plasma.** Blood from healthy donors was collected on 0.13 mol/L trisodium citrate; nine parts of blood to one part of citrate solution. The blood was then centrifuged twice at room temperature for 15 minutes at 3,000g. A third centrifugation was done at 4°C for 1 hour at 23,000g. The obtained platelet-free plasma was stored at -80°C.

**Factor IXa determination.** To follow the activation of factor IX by factor Xla, an assay was developed in which factor IXa was determined via its ability to activate factor X in the presence of Ca2+ ions, phospholipid, and factor VIIIa. Briefly, factor IXa containing samples (10 μL) was added to 35 μL of Tris-buffer pH 7.9 (50 mmol/L Tris-HCl, 175 mmol/L NaCl, 0.5 mg ovalbumin/mL) containing CaCl2 (5 mmol/L), phospholipid (20 μmol/L), bovine factor VIII (10 U/mL), and bovine factor X (0.5 μmol/L). Factor X activation was started by the addition of 10 μL of a thrombin solution (1 μmol/L) and the reaction was allowed to proceed for 90 seconds, after which 450 μL of Tris-buffer containing 20 mmol/L EDTA and 0.22 mmol/L S-2337 was added to stop the reaction and determine the amount of factor Xa formed as described previously. From the observed rate of factor Xa formation, the amount of factor IXa present in the sample was calculated using a calibration curve made with known amounts of human factor IXa as determined by titration with antithrombin III/heparin.

**Factor IXa generation in plasma.** Tris-buffer with or without heparin, 23 μL, or pentasaccharide, was added to 160 μL of citrated human plasma and incubated for 4 minutes in a flat-bottom plastic tube while stirring. Phospholipids were also added to a final concentration of 20 μmol/L. The plasma was activated by the addition of CaCl2 (20 mmol/L) and factor Xa at the concentrations indicated. The free Ca2+ concentration (4 mmol/L) was measured using a calcium-specific electrode (Radiometer, Copenhagen, Denmark) standardized with CaCl2 in Tris buffer. Titrated samples (5 μL) were removed from the incubations, diluted 200-fold in ice-cold Tris buffer and assayed for factor Xa activity.

**Analysis of factor IXa generation curves.** The analysis of the factor IXa generation curves in the presence of proteinase inhibitors was based on the following assumptions: factor IX activation by factor Xla follows Michaelis-Menten kinetics, and inactivation of factor IXa and factor Xla can both be described as simple pseudo first order reactions.

When the actual concentration of factor IX at time t is \([IX] = (IX) - (IX)] - [IXa], where \([IXa] - the concentration of free factor IXa at time t, \([IXa] - the concentration of factor IXa-inhibitor complex at time t, then the factor IXa generation curves can be described by the following set of differential equations:

\[
\frac{d[Xa]}{dt} = -k_{IXa}[Xa] \quad (1)
\]
\[
\frac{d[IXa]}{dt} = k_{IXa}[Xa] \quad (2)
\]
\[
\frac{d[IX]}{dt} = \left(\frac{k_{IXa}}{K_{IXa}}[Xa][Xa]\right) - k_{IX}[Xa] \quad (3)
\]
where \(k_{IXa}\) and \(k_{IX}\) are the pseudo first order rate constants of inhibition of factors Xla and IXa, respectively. The initial conditions are: \([Xa]_0\), is the amount of factor Xa added to the plasma, with both \([IXa]_0\) and \([IXa]_1\) zero at \(t = 0\).

The values for \(k_{IXa}/K_{IXa}\), \(k_{IXa}\), and \(k_{IX}\) were estimated by a least squares fit of the numerical solution of equations 1 through 3 to the experimentally determined factor IXa generation curve, using an iterative Gauss-Newton algorithm.

The reported errors represent the 95% confidence interval of the parameters as estimated by linearization of the nonlinear equations at the obtained parameter values.

**RESULTS AND DISCUSSION**

**Factor IXa bioassay.** We developed an assay for activated factor IXa based on the formation of a highly efficient factor X converting complex that consists of factor IXa, phospholipids, Ca2+, and activated factor VIII. Under the assay conditions used, factor IXa is the rate-limiting factor and factor Xa generation is linear in time. Thus, the amount of factor Xa generated, measured as amidolytic activity toward S-2337, is proportional with the amount of factor IXa. Figure 1 shows the calibration curve constructed from known amounts of human factor IXa. Factor IXa concentration in the sample is plotted versus the rate of factor Xa formation. Because plasma contains approximately 80 nmol/L of factor IX, factor IXa concentrations as low as 1% of total factor IX can be detected.

A number of control experiments were performed to establish that our factor IXa bioassay was suited to monitor factor IXa generation in clotting plasma. Purified human factor IX (70 nmol/L) was added to congenital factor IX deficient plasma (less than 1% factor IX), followed by the addition of purified human factor Xla (5 nmol/L), 20 μmol/L phospholipid, and CaCl2 to a concentration of 4 mmol/L free Ca2+. Although phospholipids are not required for the factor Xla-catalyzed factor IX activation reaction, they were included to let the process of clotting proceed via factor Xa and thrombin generation into fibrin formation. The plasma clotting time was 120 seconds. At timed intervals, samples were taken from the activated plasma and immediately diluted. A 200-fold dilution was sufficient to stop further factor IXa generation and inactivation. As shown in Fig 2, virtually all factor IXa added to the plasma could be
recovered as activated factor IX. Interestingly, inhibition of activated factor IX was not observed. As a control on the effects of endogenously generated factor Xa and thrombin on the factor IXa assay, factor IX deficient plasma was activated with thromboplastin. Although the plasma clotted at 45 seconds and peak values for factor Xa and thrombin were 10 nmol/L and 360 nmol/L, respectively, baseline values for the factor IXa assay were obtained. Thus, because of the 1,000-fold dilution of the plasma sample into the assay mixture, endogenous formed factor Xa and thrombin did not interfere with the factor IXa assay. Furthermore, the dilution was sufficient to avoid influence of carried-over antithrombin III (~2 nmol/L) and heparin on the rate of factor Xa formation, and thus factor IXa determination.

Factor IXa generation in plasma at varying amounts of factor XIa. We first studied plasma factor IXa generation as a function of the factor XIa concentration. We already noted that factor IXa activity was not neutralized when generated in factor XIa-activated plasma. Figure 3 clearly shows that on the addition of factor XIa to plasma, plateau levels of factor IXa activity were reached. This finding contrasts with the second-order rate constant of inhibition of purified human factor IXa by antithrombin III as reported by Jordan et al.\textsuperscript{5} At the plasma concentration of antithrombin III (2.5 nmol/L) one might expect a half-life time of factor IXa activity of about 10 minutes. However, the failure to demonstrate decay of endogenous factor IXa in plasma is in accordance with the observation of McNeely and Griffith,\textsuperscript{5} who found that 5% of radiolabeled factor IXa appeared to be complexed with antithrombin III after 30 minutes of incubation of radiolabeled factor IX in contact-activated plasma. The plateau level of factor IXa activity increased with increasing amounts of factor XIa added to the plasma. Apparently, plasma inhibitors neutralize factor XIa activity at such a rate that at relatively low factor XIa concentrations complete neutralization of factor XIa is achieved before all plasma factor IX is converted into factor IXa. More than 5 nmol/L factor XIa was needed to obtain a factor IXa concentration in plasma that approached its proenzyme concentration. Estimation of a global apparent rate constant of inhibition of factor XIa in plasma was obtained by fitting the model described in Materials and Methods to the set of factor IXa generation curves shown in Fig 3. Because inhibition of factor IXa was not observed, the pseudo first order rate constant of inhibition of factor XIa was held constant at a value of zero. The ratio $k_{\text{cat}}/K_m$ and pseudo first order rate constant of inhibition of factor XIa were the adjustable parameters of the model. It is clear that our model (solid lines) fits well to the experimental factor IXa generation curves. The residuals of the curves fitted to the experimental data varied randomly around 0 and were smaller than 2%. The estimated value of the $k_{\text{cat}}/K_m$ ratio was 0.29 ± 0.01 (nmol/L)$^{-1}$ min$^{-1}$. This value is in good agreement with those reported by Walsh et al\textsuperscript{19} and our laboratory\textsuperscript{20} for factor XIa-catalyzed factor IX activation in purified systems, 0.47 and 0.23 (nmol/L)$^{-1}$ min$^{-1}$, respectively. The rate constants of inhibition of factor XIa as calculated from each of the factor IXa generation curves varied between 0.9 and 0.6 minutes (Table 1). These values are in good agreement with the value that can be calculated from the data reported by Scott et al.\textsuperscript{20} Taking their second-order rate constants of inhibition of factor XIa by various proteinase inhibitors determined at 37°C and the plasma concentrations of these inhibitors, one can predict a pseudo first-order rate constant of factor XIa decay in undiluted plasma of 0.86 min$^{-1}$. In our experiments the final plasma dilution of whole plasma was 80%.

The heparin-stimulated neutralization of in situ generated factor IXa. The effects of the Fourth International
Standard for Heparin (ISH) and the synthetic pentasaccharide heparin on factor IXa generation and inhibition were studied in normal plasma activated with factor XIa (1.25 mmol/L) in the presence of 4 mmol/L free Ca$^{2+}$ and 20 μmol/L phospholipid. Whereas the quality of the fit of the mathematical model to the factor IXa activity data obtained in the absence of heparin appears to be quite acceptable (Fig 3), it was not in the presence of heparin. At the decay phase following the plasma clotting time large deviations between the experimental generation curve and the computed one were found. This finding indicates that the factor IXa decay becomes much slower once the plasma clots. Possible explanations for this phenomenon are consumption of antithrombin III caused by the generation of large amounts of thrombin (~1.0 μmol/L) and/or protection by fibrin monomers of factor IXa from inactivation by heparin-antithrombin III, as recently has been reported for thrombin.

To compare the heparin-stimulated inhibition of in situ generated factor IXa before and after fibrin formation, the following experiments were performed. Plasma was incubated with factor XIa (2 nmol/L), Ca$^{2+}$, and phospholipid as described in Materials and Methods. At the time point (4 minutes) when factor IXa activity reached a final level, ISH (0.18 μg/mL) was added and the rate of neutralization of factor IXa activity was determined as shown in Fig 4. Under these conditions, the decay of factor IXa activity was measured after clot formation (clotting time was 150 seconds). To examine factor IXa decay before the clotting time of plasma, it is necessary either to stop further factor IXa generation or to lengthen the clotting time of plasma until the decay phase of factor IXa activity could be studied without further factor IXa generation and before the plasma clotted. Figure 4 shows that factor IXa generation in factor XIa-activated plasma is not affected by hirudin. The very same levels of factor IXa activity were obtained whether hirudin was present or not. The heparin-stimulated rate of inhibition of in situ generated factor IXa was indeed faster before the clotting time of plasma than after it. Addition of 1.0 μmol/L of purified human antithrombin III to the clotted plasma did not significantly enhance the rate of the heparin-stimulated inhibition of factor IXa, indicating that consumption of antithrombin III did not lower the rate of the heparin-stimulated inhibition of factor IXa.

The reduced rate of factor IXa inhibition was also observed when clot formation was induced by factor Xa (10 nmol/L) in factor VIII deficient plasma, indicating that the possibility of protection of factor IXa from inhibition by antithrombin III/heparin as a result of complex formation with factor VIIIa and phospholipid could be ruled out (data not shown). Whether fibrinogen degradation products and/or, as recently demonstrated for heparin-stimulated inactivation of thrombin, fibrin monomer protects factor IXa from inactivation by heparin-antithrombin III remains to be established.

Factor IXa neutralization studies in the presence of hirudin (0.5 μmol/L), using the approach shown in Fig 4, were then performed to determine the pseudo first order rate constant of inhibition of in situ generated factor IXa as a function of the amount of ISH and that of synthetic pentasaccharide heparin. The amount of hirudin was sufficient to prolong the clotting time from 2.5 minutes to more than 10 minutes and was not found to influence the factor XIa-catalyzed factor IX activation in plasma.

Table 2 lists the pseudo first order rate constants of inhibition of endogenous factor IXa as determined in factor XIa-activated plasma containing varying amounts of ISH or pentasaccharide. From the linear dependency between the rate constant and the amount of heparin, rate constants of 5.2 min$^{-1}$ per μg ISH/mL and 0.76 min$^{-1}$ per μg pentasaccharide/mL were calculated. Thus, on a weight basis, ISH was seven fold more active than pentasaccharide in its stimulation of the factor IXa-antithrombin III reaction. On a molar basis, ISH (mean molecular weight [mol wt] = 14,000 and 40% antithrombin III high affinity material) is about 140-fold more active than pentasaccharide (mol wt = 1,700).

Although little is known about the interaction of factor IXa-heparin-antithrombin III, it is generally assumed that the mechanism of factor IXa inhibition resembles that of thrombin. That is, binding of antithrombin III and factor IXa to the same heparin molecule is required for rapid
Table 2. Pseudo First Order Rate Constants of Inhibition of Factors Xla and IXa in Plasma Containing Fourth ISH and Synthetic Pentasaccharide Heparin (PENTA)

<table>
<thead>
<tr>
<th>Heparin (µg/mL)</th>
<th>$k_{na}$ (min⁻¹)</th>
<th>$k_{na}$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.59 ± 0.02</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>0.46 ± 0.02</td>
<td>0.27</td>
</tr>
<tr>
<td>0.10</td>
<td>0.79 ± 0.04</td>
<td>0.49</td>
</tr>
<tr>
<td>0.18</td>
<td>0.69 ± 0.04</td>
<td>1.00</td>
</tr>
<tr>
<td>Penta</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.58 ± 0.07</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.66 ± 0.09</td>
<td>0.35</td>
</tr>
<tr>
<td>1.0</td>
<td>0.63 ± 0.12</td>
<td>0.85</td>
</tr>
<tr>
<td>2.5</td>
<td>0.63 ± 0.18</td>
<td>1.90</td>
</tr>
</tbody>
</table>

inactivation of factor IXa. Therefore, heparin fragments below mol wt = 5,000 (18 saccharides) are believed to be of insufficient length to potentiate the inhibition of factor IXa. Therefore, the stimulation of the factor IXa/antithrombin III reaction by pentasaccharide, which exerts its catalytic activity solely through a binding-induced conformational change in antithrombin III, is an interesting observation. It indicates that the so-called approximation effect is less important for factor IXa inhibition than it is for thrombin inhibition.

Effect of heparin on factor Xla-induced factor IXa generation in plasma. Next we examined the effects of the Fourth ISH and pentasaccharide on the pseudo first order rate constant of inhibition of factor XIa during factor IX activation. To this end, plasma was activated with factor XIa in the presence of varying amounts of ISH (Fig 5) and in the presence of varying amounts of pentasaccharide (Fig 6). Each set of factor IXa generation curves thus obtained was analyzed by fitting the model described in Materials and Methods to the experimental data points. In fitting the generation curves, the pseudo first order rate constant of inhibition of factor IXa was held constant at a value corresponding to the respective heparin concentration used, and independently determined as described in the previous section (Table 2). The fitted parameters thus obtained were the pseudo first order rate constant of inhibition of factor XIa and the $k_{na}/K_m$ ratio. We conclude that at the heparin concentrations used, ISH and pentasaccharide did not stimulate the inactivation of factor XIa in plasma (Table 2). Thus, although it has been reported that heparin with high affinity for antithrombin III at an amount of 1 U/mL markedly enhanced the rate of factor XIa inactivation by antithrombin III and C1-inhibitor in plasma, this was not observed at heparin concentrations in the therapeutic range of about 0.1 U/mL of unfractionated heparin.

The fitted $k_{na}/K_m$ ratios corresponding to the set of curves shown in Figs 5 and 6 were $0.331 ± 0.008$ and $0.31 ± 0.02$ (nmol/L)⁻¹ min⁻¹, respectively. These values are in good agreement with the value of $0.29 ± 0.01$ (nmol/L)⁻¹ min⁻¹ obtained from the set of generation curves at different factor XIa concentrations in the absence of heparin (Fig 3).

As judged from the “goodness of fit,” we conclude that factor XIa-induced factor IXa generation in plasma can be acceptably described by a mathematical model that assumes Michaelis-Menten kinetics of factor XIa-catalyzed factor IX activation in plasma and pseudo first order kinetics of the reactions between plasma inhibitors and factor XIa and factor IXa. Our data suggest that when plasma coagulation is started by factor XIa and heparin is present, the area under the factor IXa generation curve is dependent on the rate of inhibition of factor IXa, and thus on the amount of heparin present. The very same might be true when factor IX is activated by the factor VII(a)/tissue factor complex, because the activity of the complex is also poorly inhibited, if at all, by heparin/antithrombin III. However, it is still an open question whether the anti-factor IXa activity of unfractionated heparin, and also that of pentasaccharide, contrib-
utes significantly to the overall anticoagulant effect. It is interesting that already small amounts of unfractionated heparin (around 0.05 U/mL) dramatically reduce the total area under the factor IXa generation curve. Because the lag phase of factor VIII activation is markedly affected by heparin, reduction of the area under the factor IXa generation curve might contribute to the anticoagulant activity of heparin because less factor IXa is available to form the physiologically important factor X activating complex at the time factor VIIIa is generated.

ACKNOWLEDGMENT

We thank Dr. Hans S,ooms for the gift of human factor XIa and Dr. H. Coenraad Hemker for stimulating discussions. The excellent technical assistance of J. Franssen and R. Bleezer is gratefully acknowledged.

REFERENCES

2. Rosenberg JS, McKenna PW, Rosenberg RD: Inhibition of factor IXa by human antithrombin III. J Biol Chem 250:8883, 1975