Inactivation of Factor XIa in Human Plasma Assessed by Measuring Factor XIa-Protease Inhibitor Complexes: Major Role for C1-Inhibitor

By Walter A. Willemin, Monique Minnema, Joost C.M. Meijers, Dorina Roem, Anke J.M. Eerenberg, Jan H. Nuijens, Hugo ten Cate, and C. Erik Hack

From experiments with purified proteins, it has been concluded that factor XIa (FXIa) is inhibited in plasma mainly by α1-antitrypsin (α1AT), followed by antithrombin III (ATIII), C1-inhibitor (C1Inh), and α2-antiplasmin (α2AP). However, the validity of this concept has never been studied in plasma. We established the relative contribution of different inhibitors to the inactivation of FXIa in human plasma, using enzyme-linked immunosorbent assays (ELISAs) to quantify the formation of complexes of FXIa with α1AT, C1Inh, α2AP, and ATIII. We found that 47% of FXIa added to plasma formed complexes with C1Inh, 24.5% with α2AP, 23.5% with α1AT, and 5% with ATIII. The distribution of FXIa between these inhibitors in plasma was independent of whether FXIa was added to plasma, or was activated endogenously by kaolin, celite, or glass. However, in the presence of heparin (1 or 50 U/mL), C1Inh appeared to be the major inhibitor of FXIa, followed by ATIII. Furthermore, at lower temperatures, less FXIa-C1Inh and FXIa-α1AT complexes but more FXIa-α2AP complexes were formed. These data demonstrate that the contribution of the different inhibitors to inactivation of FXIa in plasma may vary, but C1Inh is the principal inhibitor under most conditions.

© 1995 by The American Society of Hematology.

Materials and Methods

Reagents. Kaolin was obtained from Broacel BV (Maarssen, The Netherlands) and celite from Johns Manville Celite Division (Denver, CO). Heparin was from Kabi Vitrum (Stockholm, Sweden); Tween-20 (T2), from J.T. Baker Chemicals (Phillipsburg, NJ); soybean-trypsin inhibitor (SBTI, type I-S) and leech hirudin, from Sigma Chemicals Co (St. Louis, MO); hexamethylenemethane bromide (Polybrene), from Janssen Chimica (Beerse, Belgium); and the chromogenic substrates H-D-Pro-Phe-Arg-p-nitroanilide (S-2302) and Glp-Pro-Arg-p-nitroanilide (S-2366), from Chromogenix (Mölndal, Sweden).

Pooled normal human plasma (fresh plasma) containing 10 mmol/L EDTA and 0.05% (wt/vol) Polybrene to prevent in vitro activation of the contact system, plasma samples containing EDTA alone (EDTA-plasma), and kaolin-activated reference plasma were obtained and prepared as described. Hirudin-plasma was obtained from blood collected in plastic syringes containing hirudin (10 U/mL, final concentration) after centrifuging it at 4°C for 20 minutes at 1,600g. Aliquots of plasma from individuals of several contact phase activators, heparin, and incubation temperature was studied.

From the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and the Laboratory for Clinical and Experimental Immunology, University of Amsterdam, Amsterdam: the Department of Haematology, University Hospital Utrecht, Utrecht; the Center for Hemostasis, Thrombosis, Atherosclerosis and Inflammation Research, Academic Medical Center, and Slotervaart Ziekenhuis, Department of Internal Medicine, Amsterdam; and the Department of Internal Medicine, Free University Hospital, Amsterdam, The Netherlands.

Submitted July 25, 1994; accepted November 2, 1994.

W.A.W. is supported by a fellowship from the Swiss National Foundation for Scientific Research, and J.C.M.M. by a fellowship from the Royal Netherlands Academy of Arts and Sciences. Address reprint requests to Walter A. Willemin, MD, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Dept of Autoimmune Diseases, Plesmanlaan 125, 1066 CX Amsterdam, 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.

© 1995 by The American Society of Hematology.
als with congenital deficiencies of FXI, factor XII, prekallikrein, or HK were purchased from George King Biomedical (Overland Park, KS).

Purified human FXI was obtained from Kordia Laboratory Supplies (Leiden, The Netherlands) and was stored at −70°C in 4 mMol/L acetic acid, 150 mMol/L NaCl, pH 5.3. FXI was a single band on nondenaturing and reducing sodium dodecyl sulfate (SDS)10% (w/v) polyacrylamide gel electrophoresis (PAGE; Kordia Laboratory Supplies). β-fator XIIa was prepared from purified human factor XIIIII, as described.30 All protease inhibitors were of human origin. C1Inh was obtained from Behringwerke AG (Marburg, Germany); a1AT, a2AP, and ATIII were from Calbiochem (La Jolla, CA).

Preparation of FXIa. FXIa was prepared by incubating FXI (100 nmol/L, final concentration) with β-factor XIIa (100 nmol/L, final concentration) at 37°C in 100 mMol/L Tris-HCl, 140 mMol/L NaCl, 0.1% (w/vol) Tw, pH 7.4. The activation was monitored by the appearance of FXIa amidolytic activity. When no further increase in the amidolytic activity was observed, the reaction was stopped by neutralizing β-factor XIIa with a fivefold molar excess of monoclonal antibody (MoAb) OT-2, which completely inhibits the amidolytic activity of β-factor XIIa (see below). Amidolytic activity of FXIa was measured by using the chromogenic substrate S-2366 (0.4 mMol/L) in a buffer containing 100 mMol/L Tris-HCl, 150 mMol/L NaCl, 0.1% (w/vol) Tw at pH 8.0. The FXIa preparation obtained expressed amidolytic activity against S-2302 when tested at a similar concentration as was used for S-2366 amidolytic activity measurement, indicating that the FXIa preparation contained little, if any, (pre)kallikrein.

MoAbs. MoAb XI-5 was obtained after fusion of spleen cells from mice immunized with human FXI with Sp2/O-Ag14 myeloma cells, according to a procedure described before.30 Culture supernatants were screened using a radioimmunoassay procedure in which goat-antimouse immunoglobulin antibodies coupled with Sepharose beads were used in combination with 125I-FXI. Positive clones were directed against complexed and inactivated alAT3; and MoAb XI-5 binds equally from mice immunized with human FXI with Sp2/0-Ag14 myeloma and blocks its catalytic activity.30 All MoAbs were purified from mouse ascites fluid by affinity chromatography of FXI. MoAb RII binds equally well to native, complexed, and inactive C1Inh.30 MoAb AT-15 is directed against complexed and inactivated a1AT; and MoAb AAT-III-O, against ATIII. MoAb AAP-11 recognizes inactivated and complexed a2AP.30 MoAb OT-2 is directed against the light chain of activated factor XII and blocks its catalytic activity.30 All MoAbs were purified from mouse ascites fluid by affinity chromatography on protein-A Sepharose (Pharmacia, Uppsala, Sweden). Biotinylation of antibodies using LC-biotin-N-hydroxysuccinimide ester (Pierce, Rockford, IL) was performed according to the manufacturer’s instructions.

ELISAs for FXIa-FXIIa inhibitor complexes. Similar ELISA procedures were developed to detect the different FXIa-FXIIa inhibitor complexes. The wells of microtiter plates (Dynatech, Plochingen, Germany) were incubated for 2 hours at room temperature (or overnight at 4°C) with MoAb XI-5 (100 μg per well; 3 μg/mL in 0.12 mol/L H3BO3, 0.12 mol/L KCl, 0.08 mol/L NaOH, pH 9.5). The plates were washed with phosphate-buffered saline (PBS; 10 mMol/L sodium phosphate, 140 mMol/L NaCl, pH 7.4) containing Tw 0.02% (w/vol) after each of the following incubation steps, which were all performed with 100 μL per well at room temperature. The wells were blocked for 30 minutes with either 3% (w/vol) bovine serum albumin in PBS (ELISAs for FXIa-a2AP and FXIIa-AIITIII complexes), or with 2% (v/vol) milk in PBS (ELISA for FXIa-C1Inh complexes), or with PBS containing 0.1% (w/vol) Tw and 0.2% (w/vol) gelatine (PTG; ELISA for FXIa-a1AT complexes). The wells were then incubated for 1 hour with aliquots of test samples and standards diluted in PTG containing 1% (v/vol) normal mouse serum (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) or in PBS-milk 2% (ELISA for FXIa-C1Inh complexes). Serial dilutions of kaolin-activated pooled normal human EDTA-plasma were used as standards. Then the microtiter plates were incubated for 1 hour with the respective biotinylated MoAb diluted in PTG-1% mouse serum. In the next step, FXIa-inhibitor complexes were detected by incubating the plates for 30 minutes with polymerized horseradish peroxidase bound to streptavidin (Janssen Chimica) 1 to 10,000 diluted in PBS-milk 2%, or horseradish peroxidase-labeled streptavidin (Amersham International plc, Amersham, UK) 1 to 1,000 diluted in PTG (ELISA for FXIa-a1AT complexes). Bound peroxidase was visualized by incubating the wells with 0.11 mol/L sodium acetate, pH 5.5, containing 0.1 mg/mL 3,3',5,5'-tetramethylbenzidin (Merck, Darmstadt, Germany) and 0.003% (v/vol) H2O2. Reaction was stopped with 2 mol/L sulfuric acid, and absorbance was read at 450 nm on a Multiskan plate reader (Labsystems, Helsinki, Finland).

Quantification of FXIa-FXIIa inhibitor complexes. FXIa-FXIIa inhibitor complexes were prepared by incubating FXIa (27 nmol/L or 45 nmol/L, final concentration) with the respective inhibitors (1 to 3 nmol/L, final concentrations) for 3 hours at 37°C. Heparin (final concentration, 2 U/mL) was added to the incubation mixtures containing ATIII. None of the complex mixtures showed amidolytic activity against S-2366. For the quantification of the complexes, it was, therefore, assumed that in these mixtures FXIa was completely bound to the respective protease inhibitors. The amount of the complex in the mixtures was estimated to be equal to the initial molar FXIa concentration. Throughout this report, molar concentrations of FXIa and FXIa-inhibitor complexes were expressed based on an Mr of FXIa of 80 kD, thus calculating the active sites of FXIa. The mixtures were then preincubated for 30 to 60 minutes either in fresh plasma or in buffer and thereafter measured in the respective FXIa-FXIIa inhibitor ELISAs and compared with the kaolin-activated reference plasma. This approach allowed calculation of the different FXIa-FXIIa inhibitor complexes in this reference plasma.

In vitro activation of the contact system in plasma. One volume of pooled EDTA-plasma, of EDTA-plasma from individual donors, of hirudin-plasma, or of contact factor-deficient plasmas was mixed at 37°C with 1 vol of PBS containing various amounts of either kaolin or celite (and 300 U/mL hirudin in the case of hirudin-plasma). After an incubation time of 30 minutes, activation was stopped by adding 3 vol of PBS containing 0.1 mg/mL SBTI and 0.05% (w/vol) Polybrene (stop solution). In some experiments, stop solution without Polybrene was used. Kaolin or celite were removed by centrifuging reaction mixtures for 5 minutes at 16,000 g. An activated standard plasma was prepared similarly, by activating pooled normal EDTA-plasma for 30 minutes with kaolin in PBS (5 mg/mL, final concentration). Analogous activation of EDTA-plasma with kaolin was performed in the presence of heparin at final concentrations of 1 or 50 U/mL (heparin-plasma). For glass activation, EDTA-plasma was diluted 1:1 with PBS and incubated for 1 hour at 37°C in glass tubes. Reaction was stopped as described above.

Inactivation of exogenous FXIa added to plasma. FXIa was added to EDTA-plasma, to fresh plasma containing Polybrene (0.05%, w/vol), to hirudin-plasma (mixed with an equal volume of hirudin to a final concentration of 150 U/mL), to EDTA-plasma containing hirudin (1 or 50 U/mL), to FXI-deficient plasma, or to HK-deficient plasma (final concentration of FXI ranging between 1.35 and 22.5 nmol/L). After an incubation period of 1 hour at 37°C, or after various time intervals, dilutions of the mixtures were tested in the ELISAs for the different FXIa-FXIIa inhibitor complexes. The EDTA-plasma and hirudin-plasma used in these experiments contained 5% (v/vol) of MoAb OT-2 (0.64 mg/mL), which blocks the catalytic activity of FXIa to prevent endogenous contact activation during the incubation period. Control experiments showed no generation of FXIa-FXIIa inhibitor complexes in the absence of exog-
RESULTS

ELISAs for FXIa-FXIa inhibitor complexes. In initial experiments, optimal conditions for the ELISAs to detect FXIa-FXIa inhibitor complexes were established (see Materials and Methods). Clear dose response curves were obtained when kaolin-activated reference plasma was tested, whereas background absorbance values were observed with dilutions of unactivated fresh plasma (Fig 1). The specificity of the ELISAs was initially established in experiments with purified protease-protease inhibitor complexes. Dilutions of these complexes were tested in each ELISA and compared with dose response curves of the kaolin-activated reference plasma. In all instances, the mixtures of FXIa and a given protease inhibitor yielded a maximal response only in the appropriate assay (Table 1). Furthermore, dilutions of FXIa, C1Inh, a1AT, a2AP, or ATIII alone yielded no significant response in the assays, and purified thrombin-ATIII complexes (TAT complexes) up to a concentration of 625 μg/L showed an absorbance in the range of background. In addition, comparison of the parallel dose response curves of the prepared FXIa-FXIa inhibitor complexes of known concentrations with those obtained with kaolin-activated plasma enabled us to determine the concentration of the various complexes present in the latter reference plasma (Fig 1). From five separate experiments, it was calculated that kaolin-activated reference plasma contained (mean ± SD) 13.4 nmol/L ± 2.3 FXIa-C1Inh complexes, 15.1 nmol/L ± 1.9 FXIa-a1AT complexes, 14.0 nmol/L ± 1.7 FXIa-a2AP complexes, and 2.4 nmol/L ± 0.5 FXIa-ATIII complexes. Results of further experiments were expressed as molar concentrations based on these values. To further evaluate the
ELISAs, plasmas deficient in FXI, factor XII, prekallikrein, or HK were activated with kaolin and tested for FXIa-FXIa inhibitor complexes. As shown in Table 2, generation of enzyme-inhibitor complexes was observed in prekallikrein-deficient plasma, confirming that contact activation can occur in the absence of prekallikrein via autoactivation of factor XII. However, kaolin incubation of the other deficient plasmas did not result in detectable amounts of FXIa-FXIa inhibitor complexes.

The epitope recognized by the capture antibody MoAb XI-5 is located on the heavy chain region of FXI. Thus, binding of a certain FXIa-FXIa inhibitor complex to this antibody should not be dependent on the nature of the serpin complexed to the light chain of FXIa. Even a different capturing of the various FXIa-FXIa inhibitor complexes would not have influenced the results, as this phenomenon would occur with both the kaolin-activated reference plasma and the purified complexes used for calibration of this plasma. However, capturing of a given complex could be influenced by the presence (of high amounts) of other FXIa-FXIa inhibitor complexes, eg, by displacing complexes with lower affinity. To investigate this point, FXIa-FXIa inhibitor complexes were prepared and measured in the respective ELISAs either in the absence or in the presence of equal amounts of the other complexes (34.5 nmol/L total concentration). The values observed in the absence/presence of other FXIa-FXIa inhibitor complexes were (mean of two experiments) 8.5/8.4 nmol/L, 76.9 nmol/L, 9.78/8.8 nmol/L, and 9.3/8.6 nmol/L for FXIa-C1Inh, FXIa-a1AT, FXIa-a2AP, and FXIa-ATIII, respectively, and are shown for each ELISA in Table 3.

Formation of FXIa-FXIa inhibitor complexes after in vitro contact activation of plasma. One volume of pooled EDTA-plasma, of hirudin-plasma, or of EDTA-plasma from individual donors was incubated at 37°C with 1 vol of PBS containing different amounts of either kaolin or celite, or was incubated in glass tubes. FXIa-FXIa inhibitor complexes in the mixtures were measured, and the relative distribution of FXIa between the four different inhibitors was calculated using the sum of the concentrations of FXIa-C1Inh, FXIa-a1AT, FXIa-a2AP, and FXIa-ATIII complexes as 100%. After activation of plasma with kaolin, 30% ± 5.1% of the FXIa-inhibitor complexes generated were FXIa-C1Inh; 33.4% ± 2.6%, FXIa-a1AT; 31.4% ± 3.0%, FXIa-a2AP; and 5.2% ± 0.8%, FXIa-ATIII complexes. This distribution was not dependent on the concentration of kaolin and was the same for celite- or glass-activation (Fig 2). Similar distribution patterns were observed in hirudin-plasma (Fig 2) and in individual plasma samples (data not shown). Incubation of kaolin with fresh plasma containing Polybrene 0.05% (w/v) did not result in measurable amounts of FXIa-FXIa inhibitor complexes, thus confirming that this cationic detergent prevents contact activation of plasma. However, Polybrene was important to fully recover generated FXIa-FXIa inhibitor complexes from the activating surface: the total

<table>
<thead>
<tr>
<th>Complexes</th>
<th>ELISA for FXIa-C1Inh Complexes</th>
<th>ELISA for FXIa-a1AT Complexes</th>
<th>ELISA for FXIa-a2AP Complexes</th>
<th>ELISA for FXIa-ATIII Complexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXIa + C1Inh</td>
<td>74.6 (±13.7)</td>
<td>&lt;0.09*</td>
<td>&lt;0.3*</td>
<td>&lt;1.26*</td>
</tr>
<tr>
<td>FXIa + a1AT</td>
<td>&lt;0.07*</td>
<td>66.2 (±9.3)</td>
<td>&lt;0.3</td>
<td>&lt;1.25</td>
</tr>
<tr>
<td>FXIa + a2AP</td>
<td>&lt;0.07</td>
<td>&lt;0.09</td>
<td>71.4 (±8.8)</td>
<td>&lt;1.25</td>
</tr>
<tr>
<td>FXIa + ATIII</td>
<td>&lt;0.07</td>
<td>&lt;0.09</td>
<td>&lt;0.3</td>
<td>416.7 (±77.9)</td>
</tr>
</tbody>
</table>

Purified FXIa (two preparations with 27 or 45 nmol/L, final concentration) was incubated with either C1Inh, a1AT, a2AP, or ATIII as described in Materials and Methods. Dilutions of the mixtures were then tested in the ELISAs for FXIa-FXIa inhibitor complexes and compared with a dose response curve of kaolin-activated standard plasma. Results [means (±SD) of five separate measurements] are expressed as percentage of the concentration of FXIa-FXIa inhibitor complexes present in the standard and represent the amount of formed complexes related to 10 nmol/L purified FXIa present in the initial incubation mixtures.

* Detection limit of the respective assay.

Table 2. Formation of FXIa-FXIa inhibitor complexes after in vitro contact activation of plasma. After incubation with kaolin (5 mg/mL), plasmas congenitally deficient in one of the indicated contact system proteins were tested for the presence of the various FXIa-FXIa inhibitor complexes. Results are expressed as nanomolars.

<table>
<thead>
<tr>
<th>Plasma</th>
<th>FXIa-C1Inh</th>
<th>FXIa-a1AT</th>
<th>FXIa-a2AP</th>
<th>FXIa-ATIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXI-deficient</td>
<td>&lt;0.009*</td>
<td>&lt;0.014*</td>
<td>&lt;0.041*</td>
<td>0.036</td>
</tr>
<tr>
<td>FXII-deficient</td>
<td>0.027</td>
<td>&lt;0.014</td>
<td>&lt;0.041</td>
<td>&lt;0.030*</td>
</tr>
<tr>
<td>Prekallikrein-deficient</td>
<td>15.4</td>
<td>8.3</td>
<td>5.9</td>
<td>1.6</td>
</tr>
<tr>
<td>HK-deficient</td>
<td>0.043</td>
<td>&lt;0.014</td>
<td>&lt;0.041</td>
<td>&lt;0.030</td>
</tr>
</tbody>
</table>

After incubation with kaolin (5 mg/mL), plasmas congenitally deficient in one of the indicated contact system proteins were tested for the presence of the various FXIa-FXIa inhibitor complexes. Results are expressed as nanomolars.

* Detection limit of the respective assays.
The amount of FXIa-FXIIa inhibitor complexes measured in parallel experiments decreased by fourfold when Polybrene was avoided in the stop solution to terminate kaolin activation (data not shown).

Kaolin activation of plasma in the presence of heparin. Kaolin activation of EDTA-plasma containing 1 or 50 Unit/mL heparin was performed as described above. The total amount of generated FXIa-inhibitor complexes was between 44.1 ± 1.5 nmol/L and 59.4 ± 0.8 nmol/L, respectively, and thus, approximately the same as in plasma without heparin. However, the presence of heparin resulted in a completely different distribution of FXIa between its inhibitors (Fig 3): FXIa-ATIII complexes now accounted for 29.8% to 34.5% ± 0.6%, FXIa-C1Inh complexes had increased, too, and constituted 43.3% ± 0.5% to 65.8% ± 3.8% of the FXIa-FXIIa inhibitor complexes, whereas complexes of FXIa with a1AT and a2AP were reduced, particularly at the higher heparin concentration.

Results are expressed as means (±SD).

<table>
<thead>
<tr>
<th>Detection limit</th>
<th>ELISA for FXIa-C1Inh Complexes</th>
<th>ELISA for FXIa-a1AT Complexes</th>
<th>ELISA for FXIa-a2AP Complexes</th>
<th>ELISA for FXIa-ATIII Complexes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9 pmol/L (±5)</td>
<td>14 pmol/L (±5)</td>
<td>41 pmol/L (±15)</td>
<td>30 pmol/L (±6)</td>
</tr>
<tr>
<td>Intraassay CV</td>
<td>3.3% (±1.4)</td>
<td>1.9% (±0.9)</td>
<td>4.6% (±1.9)</td>
<td>4.7% (±1.8)</td>
</tr>
<tr>
<td>Interassay CV</td>
<td>14.7% (±3.4)</td>
<td>14.6% (±3.9)</td>
<td>6.3% (±1.7)</td>
<td>15.1% (±1.5)</td>
</tr>
</tbody>
</table>

**Inactivation of exogenous FXIa in plasma.** FXIa was added to EDTA-plasma (containing MoAb OT-2) at concentrations sufficient to prevent activation of the endogenous contact proteins during incubation time), to fresh plasma (containing Polybrene to avoid in vitro contact activation), or to hirudin-plasma (containing MoAb OT-2) to yield final concentrations of 1.35 to 22.5 nmol/L. After incubation at 37°C for 60 minutes, FXIa-FXIIa inhibitor complexes were measured. The distribution of FXIa between its inhibitors in fresh plasma (Fig 4) was essentially the same as observed after kaolin-, celite-, or glass-activation of normal plasma (Fig 2), with 31.2% ± 3.5% of the formed complexes being FXIa-C1Inh complexes, 31.9% ± 2.7% FXIa-a1AT complexes, 30.8% ± 2.0% FXIa-a2AP complexes, and 6.1% ± 1.0% FXIa-ATIII complexes. However, when FXIa was incubated in EDTA-plasma (or hirudin-plasma) in the absence of Polybrene, the relative amount of FXIa-C1Inh complexes increased to 47% ± 2.4% (49.5% ± 1.3%), whereas FXIa-a1AT, FXIa-a2AP and FXIa-ATIII complexes decreased to 23.5% ± 2.1% (22% ± 0.8%), 24.5% ± 2.5% (24% ± 1.6%), and 5% (4.5% ± 0.6%), respectively. The distribution pattern of exogenous FXIa between the four inhibitors was independent of its final concentration. Moreover, the relative amounts of formed FXIa-FXIIa inhibitor complexes were in the same range when FXIa was added to congenitally FXI-deficient plasma (data not shown). No FXIa-FXIIa inhibitor complexes were generated in the absence of added FXIa.

The total amount of formed FXIa-FXIIa inhibitor complexes increased during incubation time and reached a maximum at 60 minutes (Fig 5). The relative amounts of FXIa complexed to ATIII and a2AP were similar for all incubation
times, ranging from 4% to 6% and from 24.6% to 30.4%, respectively, whereas the contribution of C1 Inh to inactivation of heparin on the functional activity of AT III, and of heparin and HK on the inactivation pattern of exogenously added FXIa. Therefore, FXIa (22.5 nmol/L, final concentration) was added to EDTA-plasma, hirudin-plasma (both containing MoAb OT-2 to prevent activation of the endogenous contact proteins during incubation time), or fresh plasma (containing Polybrene to avoid in vitro contact activation). After incubation at 37°C for 60 minutes, FXIa-FXIa inhibitor complexes were measured. The total amount of the complexes is indicated on the left, and the percentages of the different FXIa-FXIa inhibitor complexes are shown (see legend to Fig 2).

Effect of heparin and HK on the distribution of exogenous FXIa between its inhibitors. Considering the well-known effect of heparin on the functional activity of AT III, and recent data that the presence of HK favors inhibition of kallikrein by AT III, we decided to investigate the influence of heparin and HK on the inactivation pattern of exogenously added FXIa. Therefore, FXIa (22.5 nmol/L, final concentration) was added to EDTA/OT-2-plasma containing 1 or 50 U/mL heparin (heparin-plasma), or to HK-deficient plasma, containing 0 or 1 U/mL heparin. As shown in Fig 6, inclusion of heparin dramatically changed the distribution of FXIa between its inhibitors: the amount of FXIa complexed to AT III had increased to 42% ± 1.6% (1 U/mL heparin) and to 31% ± 3.6% (50 U/mL heparin) of total FXIa complexes, and FXIa-C1 Inh complexes increased to 51.3% ± 1.5% and 67% ± 3.6%, respectively, whereas FXIa-a1AT and FXIa-a2AP complexes were strongly reduced. Similar results were obtained using FXIa at a final concentration of 3.6 nmol/L. Again, no generation of FXIa-FXIa inhibitor complexes was observed in the absence of exogenous FXIa.

FXIa added to HK-deficient plasma resulted in approximately the same distribution of FXIa-inhibitor complexes as found in EDTA-plasma (Fig 6). When heparin was added to HK-deficient plasma, again FXIa-AT III complexes remarkably increased, and FXIa-a1AT and FXIa-a2AP complexes decreased, but in a less-pronounced manner than observed in EDTA-plasma. Complexes of FXIa and C1 Inh were not affected by the addition of heparin to HK-deficient plasma.

Effect of temperature on FXIa-FXIa inhibitor complex formation. Figure 7 shows the results obtained from experiments in which FXIa (22.5 nmol/L, final concentration) was added to EDTA-plasma (containing MoAb OT-2) and incubation was performed at 37°C. After varying time intervals, further FXIa-FXIa inhibitor complex formation was prevented by diluting the samples with buffer. FXIa complexes were then measured in the ELISAs, and the total amount of generated complexes was calculated. Results are expressed as means ± SD (vertical bars) from three separate experiments.
INACTIVATION OF FACTOR XLa IN PLASMA

![Graph showing the effect of temperature on the distribution of exogenous FXIa in plasma.](image)

The ELISAs detailed in the present study proved to be specific, reproducible, and sensitive, detecting 9 to 41 pmol/L complexed FXIa (considering the dilution of plasma samples to be tested), which corresponds to activation of about 0.1% of plasma FXI. The sum of the FXIa active sites present in the different complexes in kaolin-activated reference plasma was found to be 44.9 nmol/L, corresponding to 3.6 μg/mL of FXI. This is in the range of the amount of FXI present in normal plasma (about 3 to 6 μg/mL), assuming that each active site of both 80 kD subunits of FXIa can be inactivated by proteinase inhibitors. Indeed, experiments performed using an ELISA system based on MoAb II (against C1Inh) coated to the microtiter plate and MoAb AT-15 (against complexed a1AT) as detection antibody showed that FXIa was complexed with two different inhibitors, probably each binding to one of the two light chains (data not shown). Thus, FXIa complexed to two different inhibitors will be detected in two different ELISAs. This is in agreement with reports that each of both active sites in FXIa can be inactivated by one inhibitor.

Inactivation of FXIa generated during contact activation by kaolin, celite, or glass in EDTA-plasma was essentially the same as that observed with exogenous FXIa added to Polybrene-containing plasma. Although the amount of FXIa-FXIa inhibitor complexes generated in these experiments was dependent on the concentrations of the respective contact activating surfaces, the relative distribution of FXIa between the four inhibitors did not change. Factor XIIa is protected against inactivation by C1Inh when bound to glass, sulfatides, or kaolin. FXIa bound to celite has also been reported to be protected from inactivation by C1Inh and a2AP. Therefore, it seems likely that in the experiments with Polybrene in the stop buffer, FXIa was dissociated from the activators as active FXIa and became subsequently complexed with its inhibitors in the presence of Polybrene, which, as was obvious from the experiments with exogenously added FXIa, may have influenced the distribution slightly. However, addition of Polybrene to stop the reaction was necessary to fully remove FXIa from the activating surface, in agreement with an earlier report suggesting that FXI remains surface-bound after activation.

Our results indicate that endogenously generated or exogenously added FXIa is inactivated in plasma in a similar way. This is in contrast with the inactivation of plasma kallikrein: the ratio of kallikrein complexed with α2-macroglobulin or C1Inh has been reported to be dependent on whether this enzyme was generated endogenously or added to plasma.

In the presence of heparin, FXIa was mainly inhibited by C1Inh and ATIII. Using plasma containing 1 U/mL heparin (high therapeutic dose) or 50 U/mL heparin (suprapharmacologic levels of heparin), we found a six- to eightfold increase of the relative contribution of ATIII to inactivation of FXIa in plasma. This effect of heparin was observed with different FXIa concentrations (3.6 and 22.5 nmol/L) and was independent of whether FXIa was added exogenously or generated during kaolin activation of plasma (Figs 3 and 6). These results correspond with those obtained using purified sys-

DISCUSSION

It is generally agreed that in the absence of heparin, a1AT accounts for 65% to 70% of the FXIa inhibitory activity of human plasma, whereas other inhibitors are considered to be less important. However, it was emphasized that these conclusions are based mainly on experiments with purified proteins and, therefore, may be of little relevance in determining the physiologic inhibitors of activated FXI. Analysis of the formation of complexes between FXIa and its possible inhibitors in plasma may better reflect the inactivation of FXIa in plasma. However, no extensive studies in plasma were performed until now, and methods to measure these complexes were only partially available. Therefore, we developed immunoassays for the detection of complexes of FXIa and a1AT, C1Inh, a2AP, or ATIII, respectively. Using these assays, we present evidence that C1Inh is not only the predominant inhibitor of factor XIIa but also the main inhibitor of FXIa, contributing 47% to FXIa inactivation in EDTA-plasma, followed by a2AP (24.5%), a1AT (23.5%), and ATIII (5%). The same results were obtained using plasma containing hirudin instead of EDTA (which affects the concentration of divalent cations), thereby suggesting that the pattern of FXIa inactivation found may also represent the in vivo situation.

The ELISAs detailed in the present study proved to be specific, reproducible, and sensitive, detecting 9 to 41 pmol/L complexed FXIa (considering the dilution of plasma samples to be tested), which corresponds to activation of about 0.1% of plasma FXI. The sum of the FXIa active sites present in the different complexes in kaolin-activated reference plasma was found to be 44.9 nmol/L, corresponding to 3.6 μg/mL of FXI. This is in the range of the amount of FXI present in normal plasma (about 3 to 6 μg/mL), assuming that each active site of both 80 kD subunits of FXIa can be inactivated by proteinase inhibitors. Indeed, experiments performed using an ELISA system based on MoAb II (against C1Inh) coated to the microtiter plate and MoAb AT-15 (against complexed a1AT) as detection antibody showed that FXIa was complexed with two different inhibitors, probably each binding to one of the two light chains (data not shown). Thus, FXIa complexed to two different inhibitors will be detected in two different ELISAs. This is in agreement with reports that each of both active sites in FXIa can be inactivated by one inhibitor.

Inactivation of FXIa generated during contact activation by kaolin, celite, or glass in EDTA-plasma was essentially the same as that observed with exogenous FXIa added to Polybrene-containing plasma. Although the amount of FXIa-FXIa inhibitor complexes generated in these experiments was dependent on the concentrations of the respective contact activating surfaces, the relative distribution of FXIa between the four inhibitors did not change. Factor XIIa is protected against inactivation by C1Inh when bound to glass, sulfatides, or kaolin. FXIa bound to celite has also been reported to be protected from inactivation by C1Inh and a2AP. Therefore, it seems likely that in the experiments with Polybrene in the stop buffer, FXIa was dissociated from the activators as active FXIa and became subsequently complexed with its inhibitors in the presence of Polybrene, which, as was obvious from the experiments with exogenously added FXIa, may have influenced the distribution slightly. However, addition of Polybrene to stop the reaction was necessary to fully remove FXIa from the activating surface, in agreement with an earlier report suggesting that FXI remains surface-bound after activation. Thus, our results indicate that endogenously generated or exogenously added FXIa is inactivated in plasma in a similar way. This is in contrast with the inactivation of plasma kallikrein: the ratio of kallikrein complexed with α2-macroglobulin or C1Inh has been reported to be dependent on whether this enzyme was generated endogenously or added to plasma.

In the presence of heparin, FXIa was mainly inhibited by C1Inh and ATIII. Using plasma containing 1 U/mL heparin (high therapeutic dose) or 50 U/mL heparin (suprapharmacologic levels of heparin), we found a six- to eightfold increase of the relative contribution of ATIII to inactivation of FXIa in plasma. This effect of heparin was observed with different FXIa concentrations (3.6 and 22.5 nmol/L) and was independent of whether FXIa was added exogenously or generated during kaolin activation of plasma (Figs 3 and 6). These results correspond with those obtained using purified sys-
Nishikado et al. found no significant increase in FXIa-AT complex formation in patients receiving up to 20,000 U/d of heparin, whereas it diminished complexes of FXIa therapeutic levels of heparin (1 U/mL). Nevertheless, our results unequivocally show that the relative contribution of ATIII to FXIa inactivation in plasma is strongly increased in the presence of heparin. Thus, together with a recent study showing that the inactivation of kallikrein by ATIII is potentiated by heparin, our study indicates that ATIII may be an important protein in the regulation of activated contact system enzymes.

Surprisingly, heparin also enhanced the formation of FXIa-C1Inh complexes, whereas it diminished complexes of FXIa with a1AT and a2AP, respectively. This may be explained either by accelerating the inhibition of FXIa by C1Inh or by suppressing the inactivation of FXIa by a1AT and a2AP. Evidence that heparin potentiates C1Inh has been reported. The inhibitor spectrum was the same in HK-deficient plasma when compared with normal plasma (Fig 6). Therefore, we concluded that HK had no effect on the distribution of FXIa between its inhibitors. After addition of heparin to HK-deficient plasma, the observed increase in FXIa-ATIII complexes was slightly less pronounced than that in normal heparin-plasma. This may indicate a small contribution, if at all, of HK to the heparin-dependent potentiation of the inactivation of FXIa by ATIII. This finding is in contrast with the remarkable contribution of HK to acceleration of the inactivation rate of plasma kallikrein by ATIII in the presence of heparin.

Our results are somewhat in contrast with those obtained previously with purified protein systems, where it was concluded that a1AT is the predominant inhibitor of FXIa in plasma. This may be due to several reasons. First, the second-order rate constant for the inhibition of FXIa by C1Inh, which was used to predict the relative importance of C1Inh in the previous study, is three times less than was determined by others. Second, plasma components may influence the inhibitory capacity of the different inhibitors. We found in preliminary experiments that polyanions in plasma may change the distribution of FXIa between its inhibitors due to several reasons. First, the observation in the present study that Polybrene (a polycation that neutralizes polyanions) diminishes the contribution of C1Inh from 47% to 31%. The presence of polyanions in plasma was not considered in the previous study with purified proteins. A discrepancy between results obtained from purified protein systems and from plasma system has also been found for the inhibition of plasmin by C1Inh.

Apart from the four mentioned plasma protease inhibitors, other proteins were described as FXIa inhibitors. Protease nexin-2, a protein of the α-granules of platelets, was found to have inhibitory activity against FXIa, which is accelerated by heparin. In addition, a low-molecular-weight platelet inhibitor of FXIa called PEXI was recently described. In preliminary experiments (data not shown), we investigated the influence of released (α-granule) contents of platelets to the distribution of FXIa between its plasma inhibitors. Platelet-rich plasma (PRP) was stimulated with Ca-ionophore in the same manner as described for purification of protease nexin-2. When FXIa was added to supernatant plasma of activated PRP, neither the total amount of FXIa-FXIa inhibitor complexes nor the distribution of FXIa between its inhibitors was different from unactivated supernatant or from EDTA-plasma. In addition, inclusion of heparin in supernatant plasma of activated PRP did not result in a different FXIa inactivation pattern compared with EDTA-plasma containing heparin. However, more detailed studies are necessary to clarify the role of platelets in inactivation of plasma FXIa.

Finally, we demonstrated that the distribution of FXIa between its inhibitors was affected by the incubation temperature (Fig 7). At lower temperature, the relative amount of FXIa bound to C1Inh decreased, whereas FXIa-a2AP complexes increased. The decreased inhibitory capacity of C1Inh at lower temperature has been shown for the inactivation of both plasma kallikrein and activated factor XII.

In summary, the present work indicates that (1) the contribution of protease inhibitors to inactivation of FXIa in plasma appears to be different than previously assumed based on kinetic analysis of purified proteins: C1Inh may be the predominant FXIa inhibitor; and (2) heparin alters the distribution of FXIa between its inhibitors. Further studies are required to determine the relative contribution of the different protease inhibitors in the neutralization of FXIa in vivo.

REFERENCES
11. Saito H, Goldsmith GHJ: Plasma thromboplastin antecedent


43. Wiggins RC, Bouma BN, Cochrane CG, Griffin JH: Role of high-molecular-weight kinogen in surface-binding and activation of coagulation factor XI and prekallikrein. Proc Natl Acad Sci USA 74:4636, 1977

44. Scott CF, Schapira M, Colman RW: Effect of heparin on the inactivation rate of human factor Xla by antithrombin III. Blood 60:940, 1982


49. Sim RB, Arlaud GI, Colomb MG: Kinetics of reaction of human C1-inhibitor with the human complement system protease C1r and C1s. Biochim Biophys Acta 612:433, 1980


Inactivation of factor Xla in human plasma assessed by measuring factor Xla-protease inhibitor complexes: major role for C1-inhibitor

WA Wuillemin, M Minnema, JC Meijers, D Roem, AJ Eerenberg, JH Nuijens, H ten Cate and CE Hack