Human Plasma Extrinsic Pathway Inhibitor Activity: I. Standardization of Assay and Evaluation of Physiologic Variables

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An assay was standardized to measure extrinsic pathway inhibitor (EPI) activity in human plasma. Variables that could potentially influence its measurement were systematically examined. The coefficient of variation of the assay was 6.3% for the same sample assayed on different days. The linearity regression line for a plot of observed vs expected values of mixtures of plasmas with different EPI levels was Y = 1.01X – 2.7%. Single samples from 21 healthy adults under 60 years of age varied between 74% and 159% of a pooled reference plasma. The plasma level of a given individual (eight subjects) did not vary on repeat sampling over weeks to months. EPI activity was significantly lower in plasma from umbilical cord blood (64.3% ± 12.7%, n = 16) than in plasma from adults. Mean EPI activity in adults ≥60 years of age was slightly but significantly higher (112% ± 16.8%, n = 23) than in adults <60 years of age (97.2% ± 19.0%, n = 21). EPI levels in the third trimester of pregnancy were slightly higher than in nonpregnant women. Plasma EPI levels fell slightly after surgical procedures that caused fibrinogen levels to rise, which suggests that EPI is not an acute phase reactant. Administration of 1-desamino-8-d-arginine vasopressin (DDAVP) did not alter plasma EPI levels. In two patients subjected to plasmapheresis and volume replacement with albumin and isotonic saline, plasma EPI levels returned to one-half of the levels before pheresis within about one day.

When tissue factor (TF) is exposed to circulating blood, a factor VIIa/TF complex forms that can initiate coagulation through activation of factors IX and X. However, as the severe bleeding of hemophiliac patients makes clear, direct factor VIIa/TF activation of factor X cannot, under physiologic circumstances, support normal human hemostasis. An additional activator of factor X, a factor Ixa/factor VIIIa/phospholipid complex, must also be formed. The need for this additional activator may reflect the effect of an inhibitor that quenches the activity of the factor VIIa/TF complex. This inhibitor requires factor Xa as a cofactor. Thus, as factor Xa is formed during coagulation it can trigger a regulatory mechanism that shuts down further factor VIIa/TF catalyzed generation of factor Xa.

We have called this factor Xa-dependent inhibitor of factor VIIa/TF the extrinsic pathway inhibitor (EPI). Broze et al have referred to it as the lipoprotein-associated coagulation inhibitor (LACI). The inhibitor appears to be a proteinase inhibitor containing three tandem Kunitz-type inhibitory domains. Inhibition of factor VIIa/TF activity is thought to involve two steps: an initial formation of an EPI/factor Xa complex followed by the formation of a putative calcium-dependent quaternary EPI/factor Xa/factor VIIa/TF complex. Mean plasma EPI concentration is thought to be approximately 100 ng/mL. About 50% of the activity in plasma is found in the lipoprotein fraction, from which EPI has been isolated in two molecular weight forms. Two investigative groups have reported data on the measurement of plasma EPI levels in patients. We have also carried out patient studies, which will be reported in a subsequent article. As a requisite for these studies, an EPI assay previously used in this laboratory was modified and validated for reproducibility and accuracy in measuring EPI activity in human plasma. Physiologic conditions that could affect plasma EPI activity in normal subjects and patients were systematically evaluated. These data are reported herein along with data on the rate of recovery of plasma EPI activity following therapeutic plasmapheresis of two patients.

MATERIALS AND METHODS

Materials. Human factors VII, IX, and X were purified and factor VII was activated as described previously. EPI was partially purified from plasma as reported earlier; the Q sepharose fraction used in this study represented a purification of approximately 330-fold. Human brain tissue factor was purified as described and reconstituted in vesicles of phosphatidylcholine, phosphatidyethanolamine, and phosphatidylserine in a ratio of 1:1:0.3. Siaryl H-factor IX was prepared by the general technique of Van Lenten and Ashwell as described previously. Antifactor X antiserum was raised in a goat. Bovine serum albumin (BSA) was from Sigma Diagnostics (St. Louis) and Aquosol 2 scintillation fluid was from NEN Research (Boston).

Plasma specimens were obtained from normal volunteers and from patients at the UCSD Medical Center and San Diego Veterans Administration Medical Center according to protocols approved by the Human Subjects Committee of the UCSD School of Medicine. Nine volumes of venous blood were added to 1 volume of a balanced citrate anticoagulant (sodium citrate, 0.06 mol/L, and citric acid, 0.04 mol/L), and centrifuged for ten minutes at 10,000 g. Platelet-poor plasma was removed and stored at −20°C until used. A human plasma standard, prepared by pooling plasma from 23 fasting healthy volunteer donors, was assigned a value of 100% EPI activity.

Assay of EPI. The EPI assay is based upon the ability of a test sample to inhibit factor VIIa/TF catalyzed activation of factor IX in the presence but not in the absence of factor Xa. Factor VIIa/TF is allowed to form in a calcium-containing reaction mixture in which a saturating factor VII concentration results in essentially full factor VII occupancy of limiting tissue factor binding sites. The other components of the reaction mixture are: factor X, as the precursor for the factor Xa needed as a cofactor for EPI; the test sample as the source of EPI; and H-factor IX as the substrate, whose activation, determined by activation peptide release, provides the measure

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of the residual factor VIIa/TF catalytic activity in the reaction mixture. Unless otherwise specified, the final concentrations of reactants were: 0.25 μg/mL factor VII, 10 ng/mL reconstituted TF, 2.5 μg/mL factor X, between 2% and 5% test plasma (vol/vol), 5 μg/mL 3H-factor IX and 10 mmol/L CaCl2 in a final volume of 120 μL. All constituents were diluted in 0.05 mol/L Tris, 0.15 mol/L NaCl, 50 mmol/L EDTA, 50 mmol/L benzamidine, pH 7.5, containing 5 mg/mL BSA. The reaction was initiated by the addition of the calcium and was carried out at 37°C. A 25 μL subsample to 35 μL of cold fluid and counted. TCA soluble 3H/total 3H was calculated after subtracting from each value the TCA soluble 3H of a zero time sample as background. In earlier work full activation of 3H-factor IX was found to yield approximately 35% TCA soluble 3H.

Validation of the EPI assay. EPI activity in a single stored plasma sample from each of 26 persons was measured on two or more occasions. From these determinations, the average coefficient of variation of the assay for the same sample measured on different days was determined to be 6.3%.

In an additional experiment, two Q sepharose preparations of partially purified EPI (one with an activity of 163% and the other with an activity of 454%) were added in various amounts to the plasma specimen from umbilical cord blood. The resultant measured EPI activities were higher than expected (line B, Fig 2), and yielded a regression line of Y = 1.31X + 3.7%.

To confirm that the assay was sensitive only to EPI in a diluted test plasma specimen and not to materials reportedly capable of inhibiting factor VIIa/TF activity in the absence of factor Xa20,21 we omitted factor X from the reaction mixture of the assay of several randomly selected samples from each group of normal subjects and patients studied. Factor X provided by the diluted test sample was neutralized by a preliminary incubation of the test sample with polyclonal anti-factor X IgG. Factor VIIa was substituted for factor VII to assure full activation of factor VII in the

Fig 1. An example of a reference curve for the EPI assay: Incubation mixtures contained: 0.25 μg/mL factor VII, 10 ng/mL purified, reconstituted TF, 2.5 μg/mL factor X, a dilution of pooled reference plasma, 5 μg/mL of 3H-factor IX, and 10 mmol/L CaCl2. The time courses of activation of factor IX obtained with different dilutions of the reference plasma are plotted in the inset. Symbols for the inset are: (O) control buffer, (C) 1.5% plasma, (X) 3.0% plasma, (M) 4.5% plasma, (A) 6% plasma. Values for the two-hour subsamples were used to construct the reference curve. Test specimens are assayed at dilutions yielding values falling within the steep portion of the reference curve.
PHYSIOLOGIC VARIABLES AFFECTING EPI

Effect of pregnancy on EPI activity. EPI activity was measured in 38 pregnant women and analyzed according to the trimester of pregnancy. Values appeared normally distributed. Mean EPI activity in the first trimester, 82.3% ± 12.0%, n = 11, was slightly lower than in nonpregnant women but the difference was not significant. Mean EPI activity in the second trimester was 94.5% ± 20.4%, n = 11. Mean EPI activity in the third trimester, 115% ± 27.2%, n =

![Graph showing observed vs expected EPI activity](image)

Fig 2. Plots of observed vs expected EPI activity. A plasma specimen containing 172% EPI activity was mixed in increasing proportions with a cord blood plasma specimen containing 41% EPI activity. Observed values were plotted against expected values (X) and yielded the regression line Y = 1.01X - 2.7% (A). Two partially purified preparations of EPI were added in increasing amounts to the cord blood plasma and the resultant mixtures assayed for EPI activity. Observed values were plotted against expected values, (B) and (C), and yielded the regression line Y = 1.31X + 3.7% (B).

reaction mixture in the absence of factor X. No test sample assayed under these conditions contained measurable activity inhibiting factor VIIa/TF.

Evaluation of the effect of sampling variables on EPI activity. Three samples were drawn at different times on the same day from each of seven healthy volunteers. The mean values for EPI activity on these samples were: on samples drawn at 8 AM after an overnight fast, 105% ± 26.0%; on samples drawn two to three hours after a fatty breakfast, 98.4% ± 17.2%; on samples drawn at 10 PM, 102% ± 16.3%. There was no difference between fasting and postprandial samples or between morning and evening samples as determined from single factor analysis of variance (AOV) with repeated measurements.

EPI was measured on two to six occasions in eight subjects over a period of weeks to months (total of 23 samples). The mean coefficient of variation for EPI activity of different samples from the same individual was 6.3%. The values obtained in one subject measured repeatedly over 18 months were: 85%, 110%, 99%, 95%, 90% and 89%.

Eight aliquots of the pooled reference plasma were thawed by warming at 37°C for three minutes and then frozen again by being placed in −80°C freezer. This was repeated ten times over several hours. Such repeated freezing and thawing did not affect the measured EPI activity of the samples: EPI activity before freezing and thawing, 100%; EPI activity after freezing and thawing ten times, 103%.

Effect of age on normal ranges of EPI activity. EPI activity was measured in 21 adults between the ages of 18 and 60 years (Fig 3). Activity ranged from 74% to 159% of the pooled reference plasma and the values appeared to be normally distributed with one possible outlier. The mean value was 97.2% ± 19.0%. There was no difference between values for men, 98.1% ± 15.9%, n = 8, and for women, 96.6% ± 21.2%, n = 13 (Student's t test, P > .05).

Plasma samples obtained from blood from 16 umbilical cords (normal full term deliveries) had a mean EPI activity of 64.3 ± 12.7%, which was significantly lower than the mean EPI activity of plasma from adults aged 18 to 60 years (single factor AOV followed by Dunnett's test, P < .01). EPI activity was also measured in plasma obtained from 23 healthy adults over the age of 60 years. The mean EPI activity, 112% ± 16.8%, was significantly higher than for adults aged 18 to 60 years (single factor AOV followed by Dunnett's test, P < .01).

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EPI activity following administration of 1-desamino-8-d-arginine vasopressin (DDAVP), 0.03 μg/kg in 50 mL of isotonic saline was administered intravenously (IV) over 30 minutes to seven patients with von Willebrand’s disease. As expected, the levels of factor VIII activity, von Willebrand’s antigen, and Ristocetin cofactor activity were all increased significantly in plasma obtained 30 minutes after the infusion. Levels of EPI activity were unaffected (Table 1).

Evidence that EPI is not an acute phase reactant. Plasma EPI activity was measured preoperatively and one to two days postoperatively in eight patients who underwent an uncomplicated, elective surgical procedure (inguinal herniorrhaphy, three patients; transurethral prostate resection, two; prostatic biopsy, one; open reduction and internal fixation of a trimalleolar fracture, one). Fibrinogen levels, which were measured as a positive control for an acute phase reactant, rose significantly in these patients. In contrast, mean EPI level fell slightly but significantly (Table 2).

EPI levels following plasmapheresis. Serial plasma EPI levels were measured in two patients following therapeutic plasmapheresis and replacement of plasma with 5% albumin and isotonic saline. One patient had macroglobulinemia of Waldenstrom and the second patient had a neurologic disorder for which he was also receiving 80 mg of prednisone daily. The data are plotted in Fig 4. In both patients, plasma levels returned halfway to the levels before pheresis in about one day.

DISCUSSION

Assays for EPI activity are based on its ability to neutralize the catalytic activity of the factor VIIa/TF complex. The EPI assay described herein is preferred over an assay used earlier in this laboratory in that the concentration of TF limits the amount of factor VIIa/TF formed and the measurement of activation peptide release from 125I-factor IX is used to monitor residual factor VIIa/TF catalytic activity. The present assay differs from that of Bajaj et al not only in the use of reconstituted purified TF but in avoiding a sequence in which factor VIIa/TF is formed, disassociated with EDTA, and then formed again by addition of calcium. The assay differs in a fundamental way from the assay Sandset et al developed for patient studies. In their assay factor VII limits the amount of factor VIIa/TF complex that can be formed and the test samples must be heated to neutralize plasma factor VII activity.

All assays of EPI activity described to date are technically exacting, and the present one, in which only purified reagents are used, is certainly no exception. However, the present assay is reproducible, with a coefficient of variation of 6.3% for the same sample assayed on different days, and accurately measures EPI activity in human plasma over a wide range. Thus, mixtures in various proportions of a cord blood plasma specimen containing 41% EPI activity

Table 1. Extrinsic Pathway Inhibitor Levels Do Not Change After DDAVP Administration

<table>
<thead>
<tr>
<th>Measurement*</th>
<th>Before DDAVP†</th>
<th>After DDAVP†</th>
<th>Change‡</th>
<th>P Value§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIII</td>
<td>69 ± 19</td>
<td>219 ± 53</td>
<td>150 ± 64</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Von Willebrand antigen</td>
<td>66 ± 20</td>
<td>146 ± 18</td>
<td>80 ± 26</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Ristocetin cofactor</td>
<td>64 ± 15</td>
<td>150 ± 30</td>
<td>86 ± 23</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>EPI</td>
<td>81 ± 8</td>
<td>82 ± 10</td>
<td>1 ± 7</td>
<td>&gt;.05</td>
</tr>
</tbody>
</table>

*Values are the percent activity of reference plasma.
†Group means ± SD: seven subjects.
‡Mean ± SD of differences.
§Paired Student’s t test.

**Table 2. Evidence That Extrinsic Pathway Inhibitor Is Not an Acute Phase Reactant**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Before Surgery*</th>
<th>After Surgery*</th>
<th>Change†</th>
<th>P Value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>(mg/dL) 351 ± 101</td>
<td>482 ± 118</td>
<td>106 ± 14</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>EPI (%)</td>
<td>106 ± 14</td>
<td>98 ± 12</td>
<td>-10 ± 9</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>

*Group means ± SD: eight subjects.
†Means ± SD of differences.
‡Fibrinogen, one-tailed paired Student’s t test; EPI, two-tailed paired Student’s t test.

The present assay is similar to the assay used by Bajaj et al in that the concentration of TF limits the amount of factor VIIa/TF formed and the measurement of activation peptide release from 125I-factor IX is used to monitor residual factor VIIa/TF catalytic activity. The present assay differs from that of Bajaj et al not only in the use of reconstituted purified TF but in avoiding a sequence in which factor VIIa/TF is formed, disassociated with EDTA, and then formed again by addition of calcium. The assay differs in a fundamental way from the assay Sandset et al developed for patient studies. In their assay factor VII limits the amount of factor VIIa/TF complex that can be formed and the test samples must be heated to neutralize plasma factor VII activity.

All assays of EPI activity described to date are technically exacting, and the present one, in which only purified reagents are used, is certainly no exception. However, the present assay is reproducible, with a coefficient of variation of 6.3% for the same sample assayed on different days, and accurately measures EPI activity in human plasma over a wide range. Thus, mixtures in various proportions of a cord blood plasma specimen containing 41% EPI activity
with an adult plasma specimen containing 172% EPI activity gave observed EPI levels virtually identical to expected EPI levels (Fig 2).

However, when EPI partially purified from the apoproteins of the whole lipoprotein fraction of plasma was added to cord blood plasma, observed values for EPI activity were 30% higher than expected values (Fig 2). The latter were calculated from the activity of the partially purified EPI as established by repeated assay after dilution (1/33 to 1/1,000) only in buffer. Therefore, the difference between observed and expected values could only have resulted from the EPI preparations being initially diluted in cord blood plasma before being diluted further in buffer for assay. Lacking both an EPI preparation purified to homogeneity and a sensitive assay for EPI antigen, we have not attempted to investigate mechanisms whereby a plasma milieu might augment the activity of a semi-purified EPI preparation.

At present, it would seem prudent to use pooled plasma as a reference standard when measuring EPI activity in plasma samples but to use an (at least) partially purified EPI preparation as a reference standard when measuring the EPI activity of partially purified plasma apoprotein fractions.

Plasma samples from patients for EPI assay cannot always be controlled for the time of day that the blood is drawn, for whether the patient is fasting or postprandial, and for whether the sample has been previously frozen and thawed. It was therefore reassuring to establish that these variables did not affect plasma EPI activity as measured in the present assay.

Whereas a range of 74% to 159% was found for single samples from 21 normal adults under the age of 60 years, the variability of measurements on repeated samples drawn over weeks to months from eight of these individuals did not exceed the variability of a single sample assayed on different days (coefficient of variation for each coinidentally 6.3%). The wide range of plasma EPI levels found on measuring single samples from a group of healthy persons apparently reflects persisting differences in plasma EPI levels between different normal individuals.

The lowest value, 74%, that we found for plasma EPI activity in 21 normal adults under the age of 60 years corresponds closely to the lowest value, 72%, that Bajaj et al found in 25 “healthy volunteers”. Therefore, it seems reasonable to accept a value of 70% of a pooled reference plasma as the lower limit for the normal range of plasma EPI activity for adults under the age of 60 years. The lowest value that we found for 23 adults over 60 years old was 84%, which suggests to us that the lower limit of the normal range for elderly adults should be set at 80% of a pooled reference plasma prepared from younger adults. The lowest value found in 16 plasma samples from umbilical cord blood from normal newborns was 41% of our reference plasma. The other known natural inhibitors of blood coagulation, antithrombin III, and protein C and its cofactor protein S, are also significantly below adult values in the newborn.

Although plasma EPI values in women in the first and second trimester of pregnancy did not differ from the values in nonpregnant women, the mean value of 116% for 16 women in the third trimester of pregnancy was significantly higher than the mean value of 97% for the 13 women in our 18 to 60 year old control group. Further studies in which plasma EPI levels are measured serially over the course of pregnancy would strengthen the evidence that EPI activity rises slightly in the last trimester.

Since cultured human umbilical vein endothelial cells release EPI into the culture medium, one may assume that vascular endothelium is an in vivo site of synthesis of plasma EPI. Infusions of DDAVP that caused marked rises in von Willebrand factor antigen in seven patients with von Willebrand’s disease failed to increase plasma EPI levels. This may be due to a lack of increased release EPI levels failed to rise after venous occlusion of a limb. Endothelial cells do not release EPI in response to vasoactive stimuli triggering release of stored von Willebrand factor.

Surgical procedures causing a rise in plasma levels of fibrinogen, a known acute phase reactant, resulted in a slight but significant fall in plasma EPI levels. Sandset et al also reported finding a variable fall in plasma EPI levels after surgery. One may infer from such data that the stimulus triggering increased fibrinogen synthesis in acute inflammatory states does not trigger a similarly increased rate of synthesis of EPI. However, this remains a tentative conclusion until data are available on rates of clearance of plasma EPI activity in inflammatory states.

Therapeutic plasmapheresis, in which approximately 75% of circulating plasma was removed and replaced with albumin and isotonic saline, caused plasma EPI levels to fall in two patients to about 30% of the values before plasmapheresis. However, this remains a tentative conclusion until data are available on rates of clearance of plasma EPI activity in inflammatory states.

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REFERENCES

5. Broze GJ, Warren LA, Novotny WF, Higuchi DA, Girard JJ,


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TA Warr, BJ Warn-Cramer, LV Rao and SI Rapaport