The Effect of Leukocyte Elastase on Tissue Factor Pathway Inhibitor

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Tissue factor pathway inhibitor (TFPI) is a multivalent Kunitz-type inhibitor that directly inhibits factor Xa and, in a factor Xa-dependent fashion, also inhibits the factor VIIa/tissue factor (TF) catalytic complex. The Kunitz-2 domain in TFPI is needed for the binding and inhibition of factor Xa, while the Kunitz-1 domain appears to be responsible for binding factor VIIa in a quaternary factor Xa-TFPI-factor VIIa/TF inhibitory complex. Human leukocyte elastase (HLE) proteolytically cleaves TFPI between threonine-87 and threonine-88 within the polypeptide that links the Kunitz-1 and Kunitz-2 domains in the TFPI molecule. HLE treatment not only affects the ability of TFPI to inhibit factor VIIa/TF, but also dramatically reduces its inhibition of factor Xa. Both purified HLE and stimulated neutrophils regenerate TF activity from a pre-formed factor Xa-TFPI-factor VIIa/TF inhibitory complex. Kinetic analysis suggests that HLE cleavage does not affect the affinity of the initial encounter interaction between factor Xa and TFPI, whereas it markedly reduces the affinity of the final factor Xa:TFPI complex with K (final) values for untreated and HLE-treated TFPI of 58 pmol/L and 4.4 nmol/L, respectively. Thus, an epitope in the amino-terminal region of TFPI or a conformation of the TFPI molecule that requires the presence of this region is needed in concert with the Kunitz-2 domain to produce optimal inhibition of factor Xa by TFPI.

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MATERIALS AND METHODS

Factor VII-deficient, factor X-deficient, and pooled normal human plasma samples were obtained from George King Biomedical (Overland Park, KA). Spectrozyme Xa (methoxy carbonyl-o-cyclohexylglycyl-glycyl-arginine-p-nitroanilide acetate) was obtained from American Diagnostica (Greenwich, CT), and MonoPoly Resolving Medium was from Flow Laboratories (McLean, VA). Sodium [3H]borohydride was from New England Nuclear (Boston, MA), and Iodogen was obtained from Pierce Chemical.
TFPI AND ELASTASE

(Rockford, IL). Rabbit brain cephalin (RBC), cytochalasin B, methoxy-O-succinyl-Ala-Ala-Pro-Val-chloromethyl ketone (CMK), N-formyl-Met-Leu-Phe, and bovine serum albumin (BSA) were purchased from Sigma Chemical (St Louis, MO). Heparin was from Elkins-Sinn (Cherry Hill, NJ).

Proteins. rTFPI was immunoaffinity-purified from the conditioned media of mouse C127 cells that had been transfected using a bovine papilloma virus containing wild-type TFPI cDNA. The concentration of rTFPI was determined by analysis of amino acid composition; the addition of norleucine served as an internal control for recovery. Based on the predicted amino acid sequence of TFPI, rTFPI was assumed to contain 34 ASX (asparagine + aspartic acid residues). Human factor X was isolated as previously described, and activated using the factor X coagulant protein (XCP) from Russell’s Viper venom, that had been immobilized on agarose beads. XCP was purified from crude viper venom as previously described. Trypsin was purchased from Sigma Chemical. The concentrations of human factor Xa and trypsin were determined by active-site titration. HLE and cathepsin G were from Elastin Products (Pacific, MO), and antithrombin III was from Kabo Vitum (Stockholm, Sweden). Recombinant factor VIIa was from Novo-Nordisk (Gentofte, Denmark). Crude, EDTA-washed, tissue thromboplastin from human brain was used as the source of TP.

Isolation of neutrophils. Neutrophils were isolated from freshly drawn, heparinized (final, 5 U/mL) blood using Mono-Poly Resolving Medium as described by the manufacturer. Their purity was routinely 90% to 95%, and cell viability was greater than 95% as assessed by Trypan blue exclusion. Neutrophils were treated with cytochalasin B and stimulated with f-Met-Leu-Phe to release HLE.

Effect of HLE on TFPI. rTFPI 140 μg/mL (3.3 μmol/L) was incubated with HLE 1.33 μg/mL (47 μmol/L) in 0.1 mol/L NaCl, 0.05 mol/L Tris-Cl, pH 7.5, at room temperature. At the specified times, 15 μL samples were removed and added to 5 μL of a solution containing CMK (final concentration, 385 μmol/L). Subsamples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, bovine factor Xa ligand blotting, and assayed for residual TFPI activity against trypsin, factor Xa, and factor VIIa/TF as described below.

TFPI inhibition of trypsin. Trypsin (0.5 nmol/L) was incubated with the sample of rTFPI (final concentration, 2.0 nmol/L) in TBSA (0.1 mol/L NaCl, 0.05 mol/L Tris-Cl, pH 7.5, with 1 mg/mL bovine serum albumin) at room temperature. After 15 minutes, the remaining trypsin activity was determined by measuring the initial ΔA 405/minute following the addition of Spectrozyme Xa (final concentration, 100 μm). Relative TFPI activity was determined by comparison of the result to a standard curve constructed using rTFPI that had been treated in the same fashion but in the absence of HLE.

TFPI inhibition of factor Xa. Each sample of rTFPI (final concentration, 5.0 nmol/L) was incubated with human factor Xa (1 nmol/L) in TBSA at room temperature. After 30 minutes, 60 μL of this mixture was added to a fibrometer cup containing 60 μL CaCl₂ (25 mmol/L) and 60 μL RBC prepared following the manufacturer’s instructions, and incubated at 37°C. After 30 seconds, 60 μL of factor X-deficient human plasma was added and the time to clot formation determined with a fibrometer (BBL, Cockeysville, MD). Remaining TFPI activity was determined by comparison of the clotting time to a standard curve constructed with rTFPI that had been treated in the same fashion but in the absence of HLE.

TFPI inhibition of factor VIIa/TF. Factor VIIa/TF inhibition by rTFPI was determined using a modification of the previously described factor X activation peptide release assay. Twenty-five-microliter samples of TFPI (2.5 nmol/L) in TBSA were incubated with 50 μL of a mixture containing factor VIIa (8 nmol/L), TF (1%), CaCl₂ (8 mmol/L), and factor X (33 nmol/L) at room temperature. After 30 minutes, 25 μL [sia-IH]-factor X (330 nmol/L) was added and exactly 10 minutes later 200 μL of ice-cold 7.5% trichloroacetic acid (TCA) was added to stop the reaction. The mixtures were placed on ice. Following centrifugation (12,000 × g, 3 minutes), the supernatants were analyzed for “H-activation peptide release. Remaining TFPI activity was determined by comparison of the activation peptide release to a standard curve constructed with rTFPI that had been treated in the same fashion but in the absence of HLE.

Coagulation assays. A modified TF inhibitor assay was used to measure TF activity. Samples were first diluted 1:1 with 4 μmol/L antithrombin III and incubated 30 minutes at room temperature. Fifty microliters of a 1:50 dilution of this mixture in TBSA with 5 mmol/L CaCl₂ was added to a fibrometer cup containing 50 μL factor VIIa (20 nmol/L), 50 μL CaCl₂ (25 mmol/L), and 50 μL factor X (170 nmol/L), and incubated at 37°C. After 1 minute, 50 μL of a mixture containing 10 parts factor X-deficient plasma and one part stock RBC was added and the time to clot formation determined in a fibrometer. Relative TF activity was determined by comparison of the clotting time to a standard curve constructed using samples derived from initial reaction mixtures containing various concentrations of TF and not rTFPI. HLE at the concentrations tested did not affect TF activity.

Factor Xa activity was determined by adding a 60-μL sample in TBSA to 60 μL RBC and 60 μL CaCl₂ (25 mmol/L) in a fibrometer cup and incubated at 37°C. After 30 seconds, 60 μL of factor X-deficient plasma was added and the time to clot formation determined in a fibrometer. Factor Xa activity was determined by comparison of the clotting time to a factor Xa standard curve.

Factor VIIa activity was determined by adding a 60-μL sample in TBSA to 60 μL CaCl₂ (25 mmol/L) and 60 μL TF (1%) in a fibrometer cup and incubated at 37°C. After 30 seconds, 60 μL of factor VII-deficient plasma was added and the time to clot formation determined in a fibrometer. Factor VIIa activity was determined by comparison of the clotting time to a factor VIIa standard curve.

Estimation of Kᵢ (initial). To estimate the affinity of the encounter complex between factor Xa and rTFPI and HLE-cleaved TFPI, the initial degree of factor Xa inhibition was determined by examination of progress curves for the ΔA 405/minute within the first minute in the presence of varying concentrations of the substrate, Spectrozyme Xa. Nine hundred-microliter mixtures containing the rTFPI sample (5 mmol/L) and Spectrozyme Xa (50, 100, 200, and 500 μmol/L) were made in TBSA. The reaction was started by the addition of 100 μL factor Xa (final, 0.5 nmol/L) and the absorbance at 405 nm was continuously recorded. A plot (Lineweaver-Burk) of the reciprocal of the velocity (ΔA 405/minute) versus the reciprocal of the initial substrate concentration yielded the Kᵢ. Kᵢ values were then derived from the equation: Kᵢ = K₅/[(K₅+Kₐ) - Kₐ], where I = rTFPI initial concentration and the K₅ of Spectrozyme Xa for factor Xa was determined independently to be 130 μmol/L.

Determination of Kᵢ (final). The Kᵢ (final) for rTFPI and HLE-cleaved rTFPI were determined using the method of Bieh. Mixtures containing factor Xa (1 nmol/L) and rTFPI samples (0, 0.5, 1.0, 1.5, and 2.0 nmol/L) were incubated at room temperature in 900 μL TBSA. After 60 minutes, 100 μL Spectrozyme Xa (final concentration, 100 μmol/L) was added and the absorbance at 405 nm was continuously monitored until the ΔA 405/minute was constant. A plot (Eaaz-Snedman) of 1/(1 - α) versus 1/α was constructed, where α = the fractional protease activity remaining, and the slope of the line yielded Kᵢ. Kᵢ values were derived by correcting for
the concentration of the substrate, Spectrozyme Xa, using the equation: 

\[ K_c = K_{\text{app}}/[1 + (S/K_c)]. \]

The \( K_c \) (final) of HLE-cleaved rTFPI against factor Xa was confirmed using a Dixon plot. Factor Xa (1 nmol/L) and rTFPI (0, 1, 2, 4, and 8 nmol/L) were incubated in 900 μL TBSA at room temperature. After 60 minutes, 100 μL of Spectrozyme Xa (final concentrations, 50, 100, and 200 nmol/L) was added and the absorbance at 405 nmol/L was monitored until the ΔA/min was constant. The reciprocal of the velocity (ΔA/min) was plotted versus initial rTFPI concentration and ~K, derived from the point of intersection of the lines generated with each concentration of Spectrozyme Xa.

**Other methods.** Immunoblotting was performed as previously described using a primary antibody raised in a rabbit against a synthetic peptide (DSEEDEDHETT), which corresponds to amino acids 1 to 10 of the TFPI molecule. The peptide was cross-linked to keyhole limpet hemocyanin using N-succinimidyl bromoacetate before its use as an immunogen, and antibodies were raised in a rabbit using the method of Vaitukaitis. Ligand blotting using 125I-bovine factor Xa was performed as previously described and SDS-PAGE was performed using the method of Laemmli. Amino-terminal sequencing was performed using electroblotted samples as previously described.

**RESULTS**

**Cleavage of TFPI by HLE.** Incubation of rTFPI with a 1:70 molar concentration of HLE produces limited proteolytic cleavage in the TFPI molecule. SDS-PAGE analysis of unreduced samples from the reaction shows the time-dependent reduction in size of the major rTFPI band from 42,000 to 34,000 molecular weight with the simultaneous appearance of lower molecular weight bands with apparent molecular weights between 8,500 and 12,500 (Fig 1A). On similar analysis of reaction samples following reduction with 5% 2-mercaptoethanol, the 34,000 molecular weight band is again seen, but the lower molecular weight bands migrate between 5,700 and 8,000 (not shown). This suggests additional HLE cleavage site(s) within the disulfide-bonded structure of the Kunitz-1 domain. A polyclonal antibody raised against a synthetic peptide based on the amino-terminus of TFPI (residues 1 to 10) recognizes the low molecular weight cleavage products, but fails to detect the higher molecular weight product at 34,000 molecular weight (Fig 1B). The amino-terminal sequence of the latter protein band is XLQKEKDFXFLEEDPGIXR (X = indeterminate), which matches residues 88 to 107 of the predicted TFPI amino acid sequence and is consistent with HLE cleavage occurring between amino acids threonine-87 and threonine-88 in TFPI. As indicated by the Western blot result (Fig 1B), the low molecular weight products (the doublet at 8,500 molecular weight and the band at 12,500 molecular weight) have the same amino-terminal sequence as full-length TFPI. Whether the different sizes of the bands in the low molecular weight triplet are in part related to differential posttranslational modifications within the portion of the TFPI molecule between residues 1 and 87 has not been determined.
**Effect of HLE treatment on TFPI function.** HLE cleavage of rTFPI is associated with a loss in the ability of rTFPI to inhibit factor VIIa/TF in the presence of factor Xa (Fig 2A). Whereas HLE treatment also reduces the ability of rTFPI to inhibit factor Xa, it had no effect on the inhibition of trypsin by rTFPI, which is also mediated predominantly through the Kunitz-2 domain (Crecelius and Broze, unpublished data) (Fig 2A). Ligand blot analysis using [125I]-bovine factor Xa (Fig 2B) shows continued binding of factor Xa by the 34,000 HLE cleavage product of rTFPI, although of much lower intensity than the binding of factor Xa to full-length rTFPI. The reduction in the affinity of cleaved rTFPI for factor Xa can best be appreciated by comparing the results of protein staining and ligand blotting for the sample obtained after 60 minutes of HLE treatment (Fig 1A, lane 7; Fig 2B, lane 6, respectively).

The kinetics of factor Xa inhibition by TFPI appears to conform to the scheme typical of other Kunitz-type inhibitors:

\[
E + I \rightarrow EI \rightarrow EI^*
\]

where \( E \) = enzyme, \( I \) = inhibitor, and \( EI \) represents the initial, immediate, encounter complex between enzyme and inhibitor with a \( K_{(\text{initial})} \) = \( k_1/k_2 \). Isomerization of the \( EI \) complex, which occurs “slowly,” leads to a “tightened” \( EI^* \) complex with a \( K_{(\text{final})} \) = \( K_{(\text{initial})} \) (\( k_3/[k_1 + k_2] \)). To more closely examine the effect of HLE cleavage on TFPI function, progress curves of the hydrolysis of the chromogenic substrate Spectrozyme Xa during the inhibition of factor Xa by rTFPI and HLE-cleaved rTFPI were performed (Fig 3). Under these conditions, the rate of Spectrozyme Xa hydrolysis (\( \Delta A_{280} \) per min) at a certain time is a measure of the remaining factor Xa activity. The immediate degrees of factor Xa inhibition produced by rTFPI and HLE-treated rTFPI, as reflected by the initial \( \Delta A_{280} \) per min, appear to be very similar (Fig 3). In additional studies, the estimated \( K_{(\text{initial})} \) was 7.6 nmol/L for both the interaction between factor Xa and uncleaved rTFPI and the interaction between factor Xa and HLE-cleaved rTFPI (see Materials and Methods). Thus, HLE-induced cleavage does not appear to effect the affinity of the initial encounter complex, \( EI \), between factor Xa and TFPI. However, progressive inhibition of factor Xa by the HLE-treated rTFPI (\( EI \rightarrow EI^* \)) is markedly reduced as compared with that produced by untreated rTFPI (Fig 3). Consistent with this observation, the \( K_{(\text{final})} \) values, determined using the method of Bieth, are 58 pmol/L and 4.4 nmol/L for untreated and HLE-cleaved rTFPI, respectively. The presence of heparin is known to enhance the inhibition of factor Xa by TFPI. When progress curves are performed in the presence of 1 U/mL heparin, the initial degree of factor Xa inhibition is increased in a comparable fashion for both forms of rTFPI, whereas progressive factor Xa inhibition is again markedly deficient for the HLE-cleaved material (Fig 3).

**Effect of HLE and stimulated neutrophils on the preformed factor Xa-rTFPI and factor Xa-TFPI-factor VIIa/TF inhibitory complexes.** rTFPI directly binds and inhibits factor Xa, whereas the mechanism for the inhibition of the factor VIIa/TF complex by TFPI is thought to involve the formation of a quaternary inhibitory complex consisting of factor Xa-TFPI-factor VIIa/TF, in which the Kunitz-2 domain in TFPI interacts with factor Xa and the Kunitz-1 domain in TFPI.
TFPI binds to factor VIIa. HLE treatment of previously formed factor Xa-rTFPI and quaternary inhibitory complexes leads to the regeneration of factor Xa and TF activity, respectively (Fig 4A). Similar regeneration of TF activity from a preformed quaternary inhibitory complex occurs when chemotactic peptide (f-Met-Leu-Phe)-stimulated, cytochalasin B–treated neutrophils are used as the source of HLE (Fig 4B). Inclusion of a specific inhibitor of HLE (MeO-Suc-Ala-Ala-Pro-Val-CH₂Cl) abrogates this effect of stimulated neutrophils, suggesting that, of the enzymes released by the neutrophils, it is HLE which is predominantly responsible for this phenomenon (Fig 4B).

Incubation of rTFPI with stimulated neutrophils produces a cleavage pattern indistinguishable from that produced with purified HLE and no proteolysis of rTFPI is detected with cathepsin G (300 nmol/L) treatment (not shown).

This regeneration of TF activity is not due to HLE-mediated proteolytic destruction of factor Xa or factor VIIa, both of which are required for the formation of quaternary inhibitory complex. Factor Xa is remarkably resistant to inactivation by HLE in the presence of physiologic concentrations of calcium (Fig 5A), and the concentrations of HLE required to inactivate factor VIIa under similar experimental conditions (presence of calcium and TF) (Fig 5B) are considerably higher than those necessary for the regeneration of TF activity (Fig 4A) and the inactivation of rTFPI (Fig 5C). Whether the HLE-mediated regeneration of factor Xa activity from the factor Xa-rTFPI complex, and whether TF activity from the quaternary factor Xa-rTFPI-factor VIIa/TF complex involves proteolysis of rTFPI between threonine-87 and threonine-88 or another site(s), remain to be determined.

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DISCUSSION

The local induction of coagulation invariably accompanies the inflammatory response to a multitude of stimuli. The agent(s) responsible for this initiation of coagulation has not been directly proven, but the presence of TF, particularly that expressed by monocytes/macrophages, at the sites of inflammation make it a likely candidate. The only known inhibitor of TF-induced coagulation is TFPI. Thus, it is of interest that the elastase (HLE) released by stimulated neutrophils, which are at high concentrations in an inflammatory exudate, inactivates TFPI and can lead to the regeneration of factor Xa and TF activity from previously formed inhibitory complexes. Whether the HLE-mediated destruction of TFPI and other coagulation inhibitors, including antithrombin III, heparin cofactor II, and C1 esterase inhibitor, that can be demonstrated in vitro actually operates in vivo to produce a local milieu favoring coagulation remains to be shown. The broad substrate specificity of HLE and other enzymes released by neutrophils suggests that they may also produce an opposing anticoagulant effect by proteolytically inactivating critical coagulation factors.

One demonstrated site of HLE proteolysis in TFPI follows threonine-87 in the intervening polypeptide between the Kunitz-1 and Kunitz-2 domains. This is a somewhat unusual site for HLE action, since the enzyme typically cleaves after valine, isoleucine, alanine, or methionine residues. Additional sites of cleavage likely exist proximal to threonine-87, because three low molecular weight cleavage products that contain the amino-terminus of TFPI were identified (Fig 1). Specifically, the presence of an additional cleavage site(s) within the Kunitz-1 domain of the TFPI molecule is suggested by the reduction in molecular weight of the lower molecular weight products following the disruption of disulfide bands with 2-mercaptoethanol. Differing heterogeneous degrees of posttranslational modification (eg, phosphorylation or O-linked glycosylation) within the TFPI molecule may also contribute to the heterogeneous molecular weights of these bands.

The loss of the ability of TFPI to inhibit the factor VIIa/TF complex after HLE treatment is consistent with previous studies suggesting that simultaneous binding of the Kunitz-1 domain to factor VIIa and the Kunitz-2 domain to factor Xa within the TFPI molecule is required for the formation of the factor Xa-TFPI-factor VIIa/TF inhibitory complex. The concomitant reduction in TFPI inhibitory activity against factor Xa was less predictable. This suggests that an epitope in the amino-terminal domain
(residues 1 to 87) of TFPI is needed for optimal binding and inhibition of factor Xa, or alternatively, that the aminoterminal region is required for a particular conformation of the TFPI molecule that facilitates its interaction with factor Xa. Interestingly, the kinetic studies show that the major effect of HLE-induced cleavage on the inhibition of factor Xa is not a reduction in the affinity of the initial, immediate factor Xa/TFPI encounter complex, EI, but instead, a marked reduction in the affinity of the final, “tightened” complex, EI*. This result is directly opposite that produced by carboxy-terminal truncation of the TFPI molecule, which is associated with a dramatic reduction in the affinity of the initial encounter interaction between factor Xa and TFPI. In summary, these studies demonstrate that considerably more of the TFPI molecule than simply the Kunitz-2 domain is involved in the inhibition of factor Xa.

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