

# Physiological Concentrations of Tissue Factor Pathway Inhibitor Do Not Inhibit Prothrombinase

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**Tissue factor pathway inhibitor (TFPI) is a Kunitz-type serine proteinase inhibitor that directly inhibits factor Xa and, in a factor Xa dependent manner, inhibits the factor VIIa/tissue factor catalytic complex. The inhibitory effect of TFPI in prothrombin activation assays using purified components of the prothrombinase complex was examined. When factor Xa is added to mixtures containing TFPI, prothrombin, calcium ions, and nonactivated platelets or factor V and phospholipids, TFPI significantly reduces subsequent thrombin generation, and the inhibitory effect is enhanced by heparin. If factor Xa is preincubated with calcium ions and thrombin-activated platelets or factor Va and phospholipids to permit**

**formation of prothrombinase before the addition of prothrombin and physiologic concentrations of TFPI (<8 nmol/L), minimal inhibition of thrombin generation occurs, even in the presence of heparin. Thus, contrary to results in amidolytic assays with chromogenic substrates, prothrombinase is resistant to inhibition by TFPI in the presence of its physiological substrate, prothrombin. Higher concentrations of TFPI (~100 nmol/L), similar to those used in animal studies testing for therapeutic actions of TFPI, do effectively block prothrombinase activity.**

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**A** CENTRAL reaction in the blood coagulation cascade is the conversion of prothrombin to thrombin by factor Xa. Factor Xa combines with calcium, phospholipids, and factor Va to form the macromolecular prothrombinase complex that enhances the rate of prothrombin activation almost 300,000-fold when compared with factor Xa alone.<sup>1,2</sup> The prothrombinase complex behaves kinetically as a single enzyme<sup>3</sup> and is thought to work most efficiently on the platelet surface where the platelet provides phospholipid and a concentrated source of partially cleaved factor V.<sup>4</sup> Importantly, when factor Xa combines with the other components of the prothrombinase complex on the platelet surface it is resistant to inhibition by antithrombin, the major plasma proteinase inhibitor of factor Xa.<sup>2,5</sup>

Tissue factor pathway inhibitor (TFPI) is a second plasma proteinase inhibitor that inhibits factor Xa, although its plasma concentration (2.5 nmol/L) is much lower than antithrombin (4.5  $\mu$ mol/L). It is an approximately 43-kD, trivalent, Kunitz-type inhibitor that directly inhibits factor Xa with the second Kunitz domain. After factor Xa is bound, it rapidly inhibits the factor VIIa/tissue factor (TF) catalytic complex with the first Kunitz domain.<sup>6</sup> The third Kunitz domain does not have a known inhibitory function. In addition to the three Kunitz-type domains, TFPI has an acidic amino-terminal region and a very basic carboxy-terminal region. A major portion of the circulating plasma TFPI is variably truncated and lacks the basic carboxy-terminal region.<sup>7</sup>

In contrast to antithrombin, TFPI appears to be an efficient inhibitor of factor Xa in the presence of the other components of the prothrombinase complex. It readily impedes clot formation in one-stage, factor Xa initiated plasma clotting assays.<sup>8</sup> In amidolytic assays using a chromogenic substrate, the rate of factor Xa inhibition by TFPI is distinctly affected by the sequential addition of components of the prothrombinase complex. In the presence of physiologic levels of calcium ions (2.5 mmol/L) the inhibitory rate is significantly slowed compared with that observed in their absence. However, the addition of phospholipids and factor Va enhances the rate of inhibition nearly to that observed in the absence of calcium.<sup>9</sup> Truncated forms of TFPI, lacking the basic carboxy-terminal region of the molecule, are much less effective inhibitors in both the clotting and the amidolytic

assays, indicating that this region, in addition to the second Kunitz domain, is required for optimal inhibition of factor Xa and the prothrombinase complex.<sup>8,10,11</sup>

To further define the role of TFPI as an inhibitor of factor Xa, its effect on prothrombin activation in mixtures containing purified components of the prothrombinase complex was examined. Contrary to the results of the chromogenic substrate assays, TFPI-mediated factor Xa inhibition in the thrombin generation assays is markedly dependent on how the reaction is started and whether factor V or factor Va is present when the reaction is initiated.

## MATERIALS AND METHODS

**Proteins and peptides.** Recombinant, full-length, human TFPI produced in *Escherichia coli* was a gift of the Monsanto Co (Chesterfield, MO). TFPI-160, an altered form of TFPI truncated after glycine 160, was produced in *E coli* and purified as previously described.<sup>12</sup> This protein contains the entire first two Kunitz domains of TFPI but lacks the third Kunitz domain and the basic carboxy-terminal region. Human factor X was isolated and activated as previously described.<sup>13</sup> Prothrombin was isolated as previously described.<sup>13</sup> Human factor V and thrombin activated factor Va were purchased from Haematologic Technologies (Essex Junction, VT). On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis ~85% of the factor V was in the single-chain 330-kD form with the remaining protein a series of high molecular weight bands ranging from 150 to 300 kD. Factors V and Va were inactivated by incubating in 5 mmol/L EDTA at 37°C for 2 hours. Inactivation was confirmed by performing one-stage factor

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Xa initiated clotting assays substituting factor V deficient plasma (George King Biomedical, Overland Park, KS) for normal pooled plasma as described later. Sheep-antihuman factor V purified IgG was purchased from Enzyme Research Laboratories Inc (South Bend, IN). Rabbit polyclonal antibodies recognizing either the amino- or carboxy-terminus of TFPI were produced as previously described.<sup>10</sup> The collagen platelet aggregation reagent was from Helena Laboratories (Beaumont, TX). Thrombin was from American Diagnostica Inc (Greenwich, CT).

### Assays

**Factor Xa-induced coagulation of plasma.** In a fibrometer (Becton Dickinson and Co, Cockneysville, MD) 50  $\mu$ L of rabbit brain cephalin, prepared as described by the manufacturer (Sigma, St Louis, MO) and diluted 10-fold into 50 mmol/L Tris-HCl pH 7.5, 100 mmol/L NaCl, 0.1% bovine serum albumin (TBSA), 50  $\mu$ L of CaCl<sub>2</sub> (25 mmol/L) and 50  $\mu$ L of factor Xa (1 nmol/L) are incubated at 37°C. After 30 seconds, 100  $\mu$ L of a 50/50 mixture of TFPI sample and normal human plasma (George King Biomedical) is added, and the degree of apparent factor Xa inhibition is determined by comparison of the clotting time to a standard curve generated by performing the assay with different concentrations of factor Xa.

**Direct activation of prothrombin by the prothrombinase complex.** In factor Xa initiated reactions, prothrombin (1.4  $\mu$ mol/L), factor V (16 nmol/L), or factor Va (3 nmol/L), rabbit brain cephalin (1:40 dilution of the stock preparation) calcium (2.5 mmol/L), and various concentrations of TFPI or TFPI-160 are mixed. Porcine intestinal heparin (0.5 U/mL) (Rugby Laboratories, Rockville Center, NY) is added when appropriate. The concentrations of prothrombin, calcium, and factor V were chosen to approximate physiologic conditions. Factor Va at 3 nmol/L is saturating. After incubating 2.5 minutes at 37°C, the reaction is initiated by the addition of factor Xa (0.1 nmol/L). Every 15 seconds 10- $\mu$ L samples are removed, diluted 100-fold into TBSA containing 5 mmol/L EDTA and assayed for thrombin activity using the chromogenic substrate Chromozyme TH (tosyl-Gly-Pro-Arg-4-nitroanilide acetate; Boehringer Mannheim, Indianapolis, IN). No detectable thrombin is produced when factor Xa, phospholipids, or factors V or Va are omitted from the reaction. Reactions initiated with a mixture of TFPI and prothrombin are performed identically except the factor Xa is incubated with the calcium, phospholipids, and factor V or Va for 2.5 minutes at 37°C before starting the reaction. In some experiments, platelets ( $5 \times 10^8$ /mL) purified from fresh whole blood by differential centrifugation as previously described,<sup>14</sup> either nonactivated, or activated with thrombin (1 U/mL) or collagen (10  $\mu$ g/mL) for 5 minutes at 23°C, are used as a source of phospholipids and factor V.

**Factor Xa amidolytic assay.** TFPI (4 nmol/L), factor V (16 nmol/L), or factor Va (16 nmol/L), phospholipids (1:40 dilution of the stock preparation), and the chromogenic substrate Spectrozyme Xa (carbonyl-D-cyclohexylglycyl-Gly-Arg-p-nitroanilide acetate, 250  $\mu$ mol/L; American Diagnostica Inc, Greenwich, CT) are mixed with TBSA containing 2.5 mmol/L CaCl<sub>2</sub> in a cuvette. The reaction is initiated by the addition of factor Xa (0.2 nmol/L) and the A<sub>405</sub> is measured continuously. In some experiments, the reaction is initiated with a mixture of TFPI and Spectrozyme Xa instead of factor Xa as described for the prothrombin activation assays. The presence of residual thrombin activity or another contaminating proteinase in the factor Va preparation that could cleave the chromogenic substrate was excluded by the following set of experiments. In reactions containing factor Va, but not factor Xa nor TFPI, cleavage of the substrate is not observed. In reactions in which only TFPI is omitted the rate of substrate cleavage does not change significantly. When factor Va, inactivated by incubation in 5 mmol/L EDTA at 37°C for

**Table 1. Inhibition of Factor Xa Initiated Coagulation of Plasma**

	Clotting Time (s)	Apparent Xa Inhibition
No inhibitor	31.5	0
2 nmol/L TFPI	40.5	50
4 nmol/L TFPI	60	78
8 nmol/L TFPI	104.5	97
80 nmol/L TFPI-160	34	30

2 hours is used the final rate of substrate cleavage is the same as that seen with calcium and phospholipids alone. Finally, hirudin does not alter the final rate of substrate cleavage, excluding contamination with prothrombin.

**Western blot analysis.** Western blot analysis after SDS-PAGE (6% or 10%) was performed as previously described<sup>6</sup> using sheep antifactor V antibodies (Enzyme Research Laboratories) or rabbit polyclonal antibodies that recognize either the amino- or carboxy-terminus of TFPI.

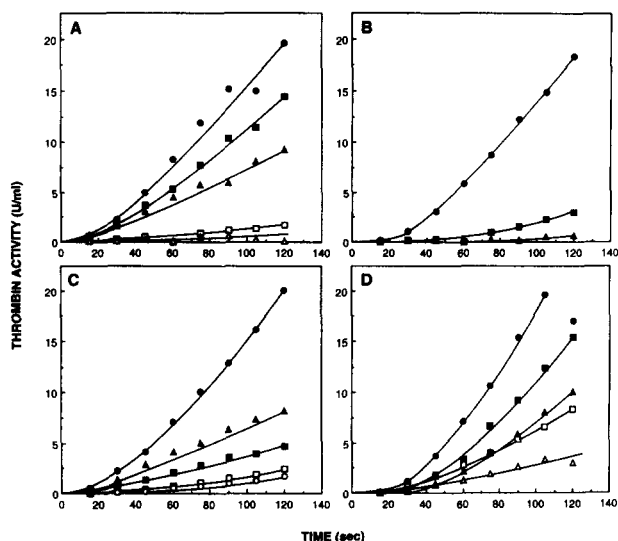
### RESULTS

#### *Comparison of TFPI Inhibition of Factor Xa in One-Stage Coagulation Assay and Prothrombin Activation Assay*

As reported in previous studies<sup>8</sup> TFPI is a potent inhibitor of factor Xa initiated coagulation in one stage plasma clotting assays with 4 nmol/L TFPI inhibiting ~80% of the apparent factor Xa activity (Table 1). On the other hand, in the prothrombin activation assay in which purified prothrombin, calcium, phospholipids, and factor Va are mixed and the reaction is initiated with factor Xa, the effect of TFPI on thrombin generation is much less pronounced. The ultimate rate of thrombin generation is reduced 20% by 4 nmol/L TFPI and 50% by 8 nmol/L TFPI (Fig 1A). TFPI-160, a truncated form of TFPI containing only the first two Kunitz domains, and other carboxy-terminal truncated forms of TFPI are much less potent inhibitors in the factor Xa initiated plasma clotting assay (Table 1).<sup>8</sup> In the prothrombin activation assay 16 nmol/L TFPI-160 inhibited thrombin generation only ~20% (data not shown). Western blot experiments failed to detect proteolytic carboxy-terminal truncation of TFPI during the course of the thrombin generation assay (data not shown).

When factor V is substituted for factor Va, TFPI is a potent inhibitor of thrombin generation (Fig 1B). When factor Va is added to 10% wt/wt of the total factor V (16-fold molar excess of factor Va over factor Xa), approximately 50% of the inhibitory activity of 4 nmol/L TFPI is lost compared to reactions containing factor V alone (Fig 1C). This result suggests that, at least in mixtures containing prothrombin, when factor Xa is associated with the other components of the prothrombinase complex it is relatively protected from TFPI inhibition.

Heparin accelerates the rate of factor Xa inhibition by TFPI in the presence of calcium when measured in amidolytic assays using a chromogenic substrate.<sup>9,15</sup> Factor Xa initiated prothrombin activation assays performed with factor Va (Fig 1A) or factor V (Fig 1D) and concentrations of heparin attainable during standard therapy (0.5 U/mL), show

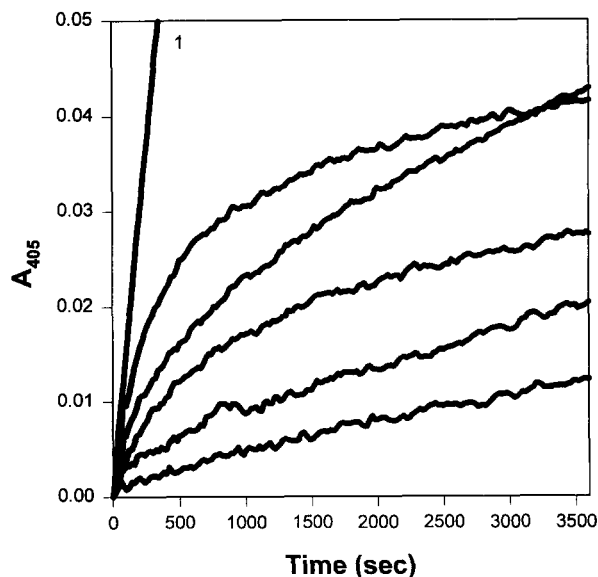


**Fig 1.** Inhibition of thrombin generation by TFPI in factor Xa initiated assays. Factor Xa (0.1 nmol/L) was added to reactions containing calcium (2.5 mmol/L), saturating phospholipids, factor Va (3 nmol/L) or factor V (16 nmol/L), prothrombin (1.4  $\mu$ mol/L), and TFPI (1 to 8 nmol/L). Samples were removed at 15-second intervals and assayed for thrombin activity. (A) Reactions initiated in the presence of factor Va: (●), No TFPI; (■), 4 nmol/L TFPI; (▲), 8 nmol/L TFPI; (□), 4 nmol/L TFPI with 0.5 U/mL heparin; (△), 8 nmol/L TFPI with 0.5 U/mL heparin. (B) Reactions initiated in the presence of factor V: (●), No TFPI; (■), 4 nmol/L TFPI; (▲), 8 nmol/L TFPI. (C) Reactions initiated in the presence of 4 nmol/L TFPI and mixtures of factors V and Va: (●), 0% Va, No TFPI; (○), 0% Va; (□), 0.1% Va; (■), 1% Va; (▲), 10% Va. (D) Reactions initiated in the presence of factor V: (●), No TFPI; (■), 1 nmol/L TFPI; (▲), 2 nmol/L TFPI; (□), 1 nmol/L with 0.5 U/mL heparin; (△), 2 nmol/L with 0.5 U/mL heparin.

that heparin greatly enhances the inhibitory activity of TFPI under the conditions of the assay.

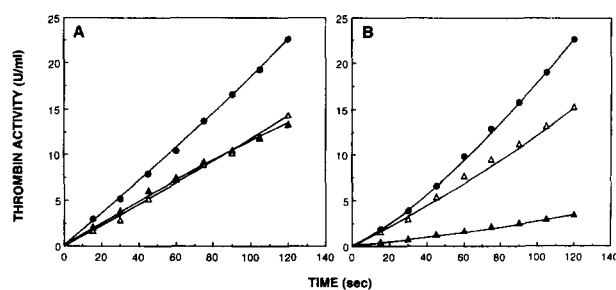
#### Order of Component Addition in Amidolytic and Prothrombin Activation Assays

The effect of factor V or factor Va on the inhibition of factor Xa by TFPI in the presence of calcium and phospholipids was reexamined using progress curves generated by the continuous measurement of the amidolytic cleavage of a chromogenic substrate by factor Xa. As shown in Fig 2, factor V enhances the inhibition of factor Xa by TFPI to a considerably greater extent than factor Va. Control experiments (see Materials and Methods) show that the differential effect of factors V and Va in these assays is not because of the presence of contaminating proteinases in the factor Va preparation. Heparin (0.5 U/mL) enhances the inhibitory effect of TFPI in the presence of both factor V and factor Va in the amidolytic assays (Fig 2). Further, in all of the amidolytic assays, nearly identical curves are obtained whether the reaction is initiated with the addition of factor Xa to the other components of the reaction or with the addition of a mixture of TFPI and chromogenic substrate to a preincubated mixture of phospholipids, calcium, factor Xa and factor V or factor Va (data not shown).



**Fig 2.** Inhibition of factor Xa by TFPI in a continuous chromogenic assay. The reactions shown were initiated by adding 0.2 nmol/L factor Xa to a mixture containing calcium (2.5 mmol/L); saturating phospholipids Spectrozyme Xa (250  $\mu$ mol/L), factor V (16 nmol/L), or factor Va (16 nmol/L); and TFPI (4 nmol/L). Nearly identical curves are obtained when the reactions are initiated with a mixture of TFPI and Spectrozyme Xa. (1), No TFPI; (2), no factor V, no factor Va; (3), factor Va; (4), factor Va with 0.5 U/mL heparin; (5), factor V; (6), factor V with 0.5 U/mL heparin.

The prothrombin activation assays (Fig 1) were repeated, this time by adding a mixture of TFPI and prothrombin to initiate the reaction after allowing the factor Xa, factor V, or Va, calcium and phospholipids to incubate for 2.5 minutes (Fig 3). As assessed by Western blot, the low concentration of factor Xa (0.1 nmol/L) used in these assays does not produce detectable activation of factor V (data not shown). Under these conditions, thrombin generation in reactions lacking heparin mirrors that produced in the factor Xa initiated experiments with TFPI inhibiting thrombin generation much better in the presence of factor V than factor Va (com-



**Fig 3.** Inhibition of thrombin generation by TFPI in TFPI/prothrombin initiated assays. The concentrations of all reactants are identical to those in Fig 1. (A) Reactions initiated in the presence of factor Va: (●), No TFPI; (▲), 8 nmol/L TFPI; (△), 8 nmol/L TFPI with 0.5 U/mL heparin. (B) Reactions initiated in the presence of factor V: (●), No TFPI; (▲), 8 nmol/L TFPI; (△), 8 nmol/L TFPI with 0.5 U/mL heparin.

pare Fig 3A and B with Fig 1A and B) In reactions containing heparin the enhancement of the TFPI-mediated inhibition of thrombin generation, present in reactions initiated with factor Xa (Fig 1A and B), is not observed in reactions initiated by the addition of a mixture of TFPI and prothrombin. Heparin does not enhance the inhibitory effect of TFPI in the presence of factor Va (Fig 3A), and it decreases the inhibition in the presence of factor V to approximately that seen in the presence of factor Va regardless of whether heparin is included in the preincubation mix (Fig 3B) or added with the TFPI/prothrombin when the reaction is initiated (data not shown). The rate of thrombin generation in the absence of TFPI is changed by heparin in neither the factor Xa nor the TFPI/prothrombin initiated reactions (data not shown).

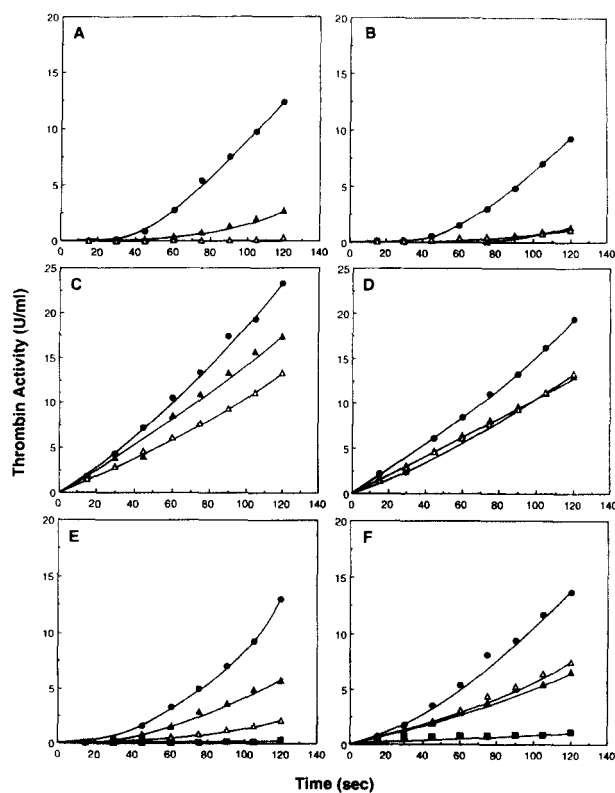
#### Platelets as a Source of Phospholipids and Factor V

Physiologically important prothrombinase activity is thought to be generated on the surface of cells, particularly platelets, which, after activation, release a partially cleaved form of factor V from their  $\alpha$  granules. Therefore, prothrombin activation assays were performed using nonactivated platelets and platelets activated through pretreatment with either thrombin or collagen. With nonactivated platelets, 8 nmol/L TFPI inhibits the ultimate prothrombin activation by about 80% (Fig 4A and B). Most of this effect is likely because of the TFPI-mediated inhibition of factor Xa that occurs during the lag period before substantial platelet activation occurs. Similar to the previous thrombin generation assays using purified factor Va and phospholipids, heparin enhances the inhibitory activity of TFPI when the reaction is initiated with factor Xa (Fig 4A) but not when it is started with a mixture of TFPI and prothrombin (Fig 4B). Moreover, the results of prothrombin activation assays using thrombin- or collagen-activated platelets, initiated with factor Xa or with TFPI/prothrombin, in the presence or absence of heparin are similar to the previous results using purified factor Va and phospholipids (Fig 4C through F), all consistent with the presence of a partially cleaved form of factor V on the platelet surface after activation.<sup>4</sup>

In recent animal studies, TFPI treatment has been shown to be efficacious in preventing disseminated intravascular coagulation and death from *E coli* sepsis in baboons,<sup>16</sup> rethrombosis after thrombolysis in dogs<sup>17</sup> and restenosis after angioplasty in pigs.<sup>18</sup> In these therapeutic trials, very high plasma levels of TFPI (>100 nmol/L) are attained. At these nonphysiologic concentrations, TFPI significantly inhibits thrombin generation on the surface of collagen activated platelets regardless of how the reaction is initiated (Fig 4E and F).

#### DISCUSSION

In amidolytic assays the inhibition of factor Xa by full-length TFPI in the presence of calcium ions is enhanced by heparin and by the addition of factor Va and phospholipids, which allows formation of the prothrombinase complex (Fig 2).<sup>9</sup> A similar effect of prothrombinase assembly on the inhibition of bovine factor Xa by tick anticoagulant peptide has



**Fig 4.** Inhibition of thrombin generation on the platelet surface by TFPI. The reactions were performed identically to those in Figs 1 and 3 except  $5 \times 10^8$  platelets/mL are used instead of factor V and phospholipids. (A) (factor Xa initiated) and (B) (TFPI/prothrombin initiated) represent reactions performed using nonactivated platelets. (C) (factor Xa initiated) and (D) (TFPI/prothrombin initiated) represent reactions performed using platelets preactivated with thrombin. (E) (factor Xa initiated) and (F) (TFPI/prothrombin initiated) represent reactions performed using platelets preactivated with collagen. For all panels: (●), No TFPI; (▲), 8 nmol/L TFPI; (△), 8 nmol/L TFPI with 0.5 U/mL heparin. In (E) and (F) (■), 100 nmol/L TFPI.

been reported.<sup>19</sup> However, in the thrombin generation assay physiologic concentrations of TFPI (<8 nmol/L) have only a minimal inhibitory effect on prothrombin activation by the preformed prothrombinase complex even in the presence of heparin (Figs 3A, 4D, and 4F). Thus, the physiologic substrate prothrombin, as opposed to the chromogenic substrate Spectrozyme Xa, competes effectively with TFPI for binding prothrombinase. It is conceivable that this is because of competition between prothrombin and TFPI for additional prothrombinase binding sites besides the catalytic site of factor Xa; for example, sites involving factor Va, phospholipids, or a distant site on the factor Xa molecule that is induced when factor Xa is bound within the prothrombinase complex.<sup>19</sup>

In thrombin generation assays containing factor Va and initiated with factor Xa, TFPI produces modest inhibition of thrombin generation and this effect is enhanced by heparin (Fig 1A and D). Similar results are obtained when activated platelets are used as the source of factor Va and phospholip-

ids (Fig 4C and E). When factor V or nonactivated platelets are substituted factor Va or activated platelets in factor Xa initiated reactions, TFPI substantially impedes thrombin generation and this effect is increased by heparin (Figs 1B, 1D, and 4A). The inhibition of thrombin production in these experiments appears to reflect TFPI-mediated inactivation of factor Xa before the generation of factor Va and/or its release from activated platelets.

The inhibition of factor Xa by TFPI is enhanced by factor V as well as factor Va in chromogenic assays (Fig 2), suggesting that an interaction between factor V and TFPI increases its ability to inhibit factor Xa. Whether this effect of the factor V preparation is because of single chain factor V itself or partially cleaved forms of factor V that contaminate the preparation is not known. Preincubation of factor Xa with factor V decreases TFPI's inhibition of prothrombin activation in the thrombin generation assay (compare Figs 1B and 3B). This likely reflects the interaction of factor Xa with trace amounts of factor Va or partially activated forms of factor V that are present in the factor V preparation or that are generated during the preincubation with factor Xa. Interestingly, the inclusion of heparin in these mixtures further diminishes the inhibitory effect of TFPI on thrombin generation (Fig 3B). The mechanism underlying this paradoxical effect of heparin is not clear, but the resultant thrombin generation mirrors that produced by preformed prothrombinase in the presence of TFPI (Fig 3A).

The data show that in the presence of prothrombin, significant inhibition of factor Xa by physiological concentrations of TFPI only occurs before the activation of factor V and the formation of the prothrombinase complex. This TFPI-mediated inhibition of factor Xa in the early stage of coagulation explains the comparable anticoagulant effect of exogenously added TFPI in plasma induced to clot by with factor Xa, tissue factor, or the X-coagulant protein of Russell's viper venom.<sup>10</sup>

These studies have used full-length TFPI, which is a potent inhibitor of factor Xa. However, a large portion of plasma TFPI (2.5 nmol/L) represents variably carboxy-terminal truncated molecules which possess much less antifactor Xa activity (Table 1).<sup>7</sup> Based on the prothrombin activation assays, normal circulating levels of full-length TFPI (possibly ~1 nmol/L) are predicted to have only a minimal effect on ultimate thrombin generation (Fig 1D). This and the fact that plasma contains alternative inhibitors of factor Xa may contribute to the observation that TFPI depletion does not sensitize animals to factor Xa/phospholipid induced intravascular coagulation.<sup>20</sup> Heparin enhances the antifactor Xa activity of TFPI<sup>9</sup> and heparin infusion raises plasma levels of TFPI twofold to fourfold,<sup>21,22</sup> apparently by releasing full-length TFPI from the endothelial surface.<sup>23</sup> Thus, plasma levels of full-length TFPI may reach 4 to 8 nmol/L with heparin treatment. Whether the inhibition of free factor Xa by TFPI in the presence of heparin adds to that of antithrombin, which in the presence of heparin is also an effective inhibitor of factor Xa outside the prothrombinase complex, is not known. However, even in the presence of heparin TFPI (<8 nmol/L) and antithrombin do not significantly

effect thrombin generation once platelet activation and prothrombinase formation have occurred (Fig 4C through F).<sup>2</sup> When used at much higher concentrations, TFPI (~100 nmol/L) effectively inhibits prothrombin activation by prothrombinase (Fig 4F), and this may contribute to the antithrombotic properties of TFPI when it is used as a therapeutic agent.<sup>15</sup>

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