Abstract—In the last decades evidence was obtained that protein S not only acts as cofactor of activated protein C (APC) in the downregulation of coagulation, but also expresses anticoagulant activity in the absence of APC. The search for the mechanism(s) underlying the APC-independent anticoagulant activity of protein S was hampered by the fact that protein S exhibited 2 seemingly identical anticoagulant activities in model systems and in plasma. Later it was shown that the anticoagulant activity of purified protein S in model systems was dependent on the concentration of phospholipid vesicles and was explained by low amounts of protein S multimers generated during purification that effectively inhibited phospholipid-dependent coagulation reactions via competition for phospholipid binding sites. Plasma does not contain multimers, and the anticoagulant activity of protein S in plasma was not affected by the phospholipid concentration but was dependent on the amount of tissue factor (TF) used for initiation of thrombin generation. This led to the discovery that protein S acts as cofactor of tissue factor pathway inhibitor (TFPI) which stimulates the inhibition of factor Xa by TFPI \( \approx 10 \)-fold. The current review describes the background of the TFPI-cofactor activity of protein S as well as the rationale for the observation that the TFPI/protein S system particularly inhibits the TF pathway at low procoagulant stimuli. (Arterioscler Thromb Vasc Biol. 2009;29:2015-2020.)

Key Words: TFPI ■ proteins ■ thrombin generation ■ extrinsic pathway ■ anticoagulant mechanisms

Anticoagulant Activities Protein S

Protein S is a vitamin K-dependent plasma protein (Mr=75 kDa) that is synthesized in the liver and in endothelial cells and which circulates in plasma both in a free form (150 nmol/L) and in complex with C4b-binding protein (200 nmol/L; for a review see1). It is well known that protein S acts as a cofactor of activated protein C (APC) in the proteolytic inactivation of blood coagulation factors Va and VIIIa, thus providing a negative feedback on coagulation and making the APC/protein S pathway essential for normal hemostasis.2–5 Homozygous deficiencies in either protein C or protein S result in severe neonatal procoagulant phenotypes, whereas heterozygous deficiencies are associated with a 10-fold increased risk of venous thrombosis.6–8

More recently it has been shown that protein S also effectively inhibits thrombin generation in plasma in the absence of APC.9 This anticoagulant property of protein S was especially observed at low TF concentrations (\( \approx 1 \) pmol/L) leading to the concept that protein S took part in regulating the initiation phase of coagulation and effectively counteracts ongoing procoagulant processes in the circulation.10 Interestingly, protein S had no effect on thrombin generation when coagulation was initiated through the intrinsic route, regardless of the amount of trigger used.5 Because of the dependence of the anticoagulant activity of protein S on the TF concentration, a new role of protein S was identified as a cofactor for TFPI in the inhibition of FXa.11

In contrast to investigations in plasma, many studies were performed in model systems using purified protein S preparations that also led to reports about APC-independent anticoagulant properties of protein S.12–16 Although the anticoagulant activities in plasma and in model systems containing purified proteins initially looked mechanistically similar, the activity of purified protein S in model systems could for the greater part be allotted to in vitro generated protein S multimers. Protein S multimers are absent in plasma17,18 and hence are not involved in the APC-independent anticoagulant activity of protein S in plasma.

Intermezzo: Protein S Multimers

When protein S was first reported to exhibit anticoagulant activity in the absence of APC,19 subsequent experiments supporting this observation were performed in model systems using purified proteins.12–14 At that time, the anticoagulant effect of protein S was explained by direct interactions of protein S with the components of the prothrombinase complex: FVa, FXa, or phospholipids that resulted in inhibition of prothrombin activation.12–14 Soon the problem arose that the APC-independent anticoagulant activity was difficult to reproduce, and that protein S preparations purified by different purification procedures had different APC-independent anticoagulant activities (Figure 1). It was observed that the activity of protein S...
proteins was dependent on the phospholipid concentration, protein S (multimers) in model systems containing purified proteins could be overcome by addition of high concentrations of phospholipids. At 1 μmol/L phospholipid, protein S1 inhibited thrombin generation 40%, and protein S2 inhibited 90%. Inset, Western blot of native PAGE of protein S1 (Mo) and protein S2 (C) showing comparable protein S monomers (Me) in protein S1 and protein S2, but more protein S multimers (Mx) in protein S2 (C).

preparations correlated with the affinity of protein S for phospholipid surfaces,29 and eventually modified protein S forms (stable multimers) were identified which were formed in protein S preparations as a result of the use of ion-exchange and affinity chromatography during purification.17,18 The multimers represented between 2% to 5% of total purified protein S and were responsible for the observed APC-independent anticoagulant activity of protein S in model systems. Protein S multimers were reported to bind to negatively-charged phospholipid surfaces with a much higher affinity (K_D < 0.5 nmol/L) than protein S monomers (K_D ~ 250 nmol/L), and the APC-independent activity of protein S preparations was explained by competition between protein S multimers and components of the prothrombinase complex for phospholipid binding sites.17 Hence, the anticoagulant activity of purified protein S could be overcome by addition of high concentrations of phospholipid vesicles (Figure 1). Furthermore, differences in activities between purified protein S preparations were explained by different protein S multimer content.17

APC-independent anticoagulant activity of protein S was also observed in plasma in which protein S was neutralized by monoclonal antibodies.9 At first it was assumed that the mechanistic basis of anticoagulant activities of protein S in model systems and in plasma was identical, but later this was shown not to be the case. In the first place, we were not able to detect protein S multimers in plasma,17,18 although this is subject to debate.21 Furthermore, the anticoagulant activity of protein S (multimers) in model systems containing purified proteins was dependent on the phospholipid concentration, and was completely abolished at high phospholipid concentrations (ie, conditions at which sufficient phospholipid surfaces were available for both protein S multimer binding and prothrombin activation15,18; Figure 1). In contrast, the APC-independent anticoagulant activity of protein S in plasma was not affected by the phospholipid concentration,10 but was strongly dependent on the TF-concentration and was particularly observed at low TF concentrations.10 This eventually pointed us in the direction of the TFPI-cofactor activity of protein S.11

TFPI
TFPI is a 276-aa Kunitz-type inhibitor (Mr=42 kDa) consisting of a negatively charged N terminus, 3 consecutive Kunitz domains, and a positively charged C-terminal tail.22 The main site of synthesis of TFPI is the endothelium.23 The first Kunitz domain binds to and inhibits FVIIa, the second binds to and inhibits FXa, and the C-terminal tail was proposed to bind to heparin-like structures on the vessel wall.24 Kunitz-3 contains a heparin binding site,25 the function of which is unknown. However, in various truncated forms of TFPI, Kunitz-3 is involved in cross-disulfide bonding between TFPI and low-density lipoprotein.26 Of total TFPI, 80% is attached to the vessel wall, whereas 20% circulates in plasma (review27). From the 20% plasma TFPI, 80% is bound to various lipoproteins and only 10% (∼0.25 nmol/L) circulates as free full-length TFPI.

TF activity is regulated by TFPI via a 2-step feedback mechanism which involves formation of a bimolecular FXa/TFPI complex that subsequently interacts with TF/FVIIa, yielding an inactive quaternary complex and resulting in termination of TF/FVIIa-catalyzed FX activation.24,28 The fact that full-length TFPI is most effective in this reaction is attributable to an intact C-terminal basic tail that interacts with anionic membrane surfaces.29,30 In this negative feedback mechanism, the initial formation a binary TFPI-FXa complex is a prerequisite for the inhibition of TF/FVIIa by TFPI.24 The concentration of full-length free TFPI in plasma is low (10% of total plasma TFPI: ∼0.25 nmol/L),31 and because TFPI is a slow tight binding inhibitor of which the K_I of the initial inhibitory complex with FXa (4 to 15 nmol/L32; Table) is much higher than the plasma TFPI concentration, this would imply that TFPI theoretically would be a weak inhibitor of TF-induced thrombin generation in plasma.

Dependence of TFPI on Protein S
In 2006 we reported that protein S acts as cofactor of TFPI11 and that the TFPI cofactor activity explains the APC-
independent anticoagulant activity of protein S in plasma. This discovery was based on the following observations: (1) in the absence of APC, protein S inhibited thrombin generation initiated via the extrinsic pathway, but not via the intrinsic pathway, (2) protein S only expressed APC-independent anticoagulant activity at low TF concentrations, (3) protein S did not express APC-independent anticoagulant activity in TFPI-deficient plasma, and (4) the anticoagulant activity of TFPI was greatly impaired in protein S-deficient plasma.

The discovery that protein S acts as a cofactor for full-length TFPI in the formation of the initial bimolecular FXa/TFPI complex explains why TFPI is an effective inhibitor in plasma.\(^\text{11}\) It was shown that protein S decreased the \(K_i\) for FXa/TFPI complex formation from 4.5 to 0.5 nmol/L. Thus, protein S makes TFPI a more efficient inhibitor by bringing the \(K_i\) of FXa inhibition in the range of the plasma concentration of full-length TFPI.\(^\text{11}\) It was concluded that through stimulation of TFPI/FXa complex formation, protein S would also enhance the formation of the quaternary TF/FVIIa/TFPI/FXa complex.\(^\text{11}\) However, more recently it was suggested that the TFPI-cofactor activity of protein S is only relevant for inhibition of free factor Xa, and not for the inhibition of TF/FVIIa by TFPI/FXa (ie, not for quaternary complex formation\(^\text{12,33}\); see also below).

It was reported that C-terminally truncated TFPI was a weak inhibitor of factor Xa\(^\text{30,34}\) (Table). In addition, truncated TFPI (1–161) was not stimulated by protein S.\(^\text{11}\) This strongly suggests that the interaction between protein S and TFPI is mediated through TFPI Kunitz-3 and the C-terminal tail. For both protein S and full-length TFPI activity it is crucial that they can bind to negatively charged phospholipids through their Glu domain and C-terminal tail, respectively.\(^\text{13,20,35}\) In addition, FXa-protein S interactions have been reported.\(^\text{6,37}\) Together with the observation that the C terminus of TFPI directly interacts with the Glu domain of FXa,\(^\text{34}\) this implies that during inhibition of FXa by TFPI/protein S at a certain moment a trimolecular complex between FXa, TFPI, and protein S on phospholipids will exist. In this review, the abbreviation TFPI will be used for full-length TFPI, unless otherwise indicated.

Supporting evidence for direct interactions between TFPI and protein S is the observation by Castoldi and coworkers that protein S and TFPI circulate as a complex in plasma (Castoldi, Simioni, Tormene, Rosing, and Hackeng, unpublished data, 2009), which could explain the covariance of protein S and TFPI in normals\(^\text{39}\) and the low levels of TFPI in protein S-deficient patients (Castoldi et al, ISTH 2009, abstract # 2338).

An illustrative example of the importance of the TFPI cofactor activity of protein S is shown in Figure 2 in which the effect of TFPI on thrombin generation in TFPI-deficient plasma and in combined TFPI-protein S-deficient plasma were compared. Reconstitution of TFPI-depleted plasma with TFPI shows a dose-dependent inhibition of thrombin generation, which is fully inhibited at 3.2 nmol/L TFPI. However, when protein S is neutralized by addition of antibodies against protein S, TFPI anticoagulant activity is severely impaired resulting in substantial thrombin generation (>50%) at 3.2 nmol/L TFPI (Figure 2).

**TFPI is a Slow Tight Binding Inhibitor**

Inhibition of FXa by TFPI proceeds through a 2-step process. In the first step, rapidly a small amount of loose complex between Kunitz-2 of TFPI and FXa is formed \((\text{FXa} - \text{TFPI})\), which results in rapid inactivation of part of the FXa. A subsequent slow rearrangement results in formation of the final tight enzyme-inhibitor complex\(^\text{24} \) \((\text{FXa} - \text{TFPI}^\ast\)\).

\[
\frac{\text{FXa} + \text{TFPI}}{\text{FXa} - \text{TFPI}} \leftrightarrow \frac{\text{FXa} - \text{TFPI}^\ast}{\text{FXa} - \text{TFPI}} \quad (a)
\]

The dissociation constant of the first rapid equilibrium is represented by \(K_i\) \((K_i = k_{-1}/k_1 = [\text{FXa}]_0/[\text{TFPI}]/[\text{FXa} - \text{TFPI}]\)) and after the subsequent slow isomerization has taken place the overall equilibrium constant becomes \(K_i^\ast\) \((K_i^\ast = [\text{FXa}]_0/[\text{TFPI}]/([\text{FXa} - \text{TFPI}] + [\text{FXa} - \text{TFPI}^\ast]))\), which is several orders of magnitude lower than \(K_i\) (Table).

The fact that TFPI is a slow inhibitor of FXa has important implications for the downregulation of the TF pathway by TFPI in plasma. TFPI anticoagulant activity in thrombin generation was only observed at low concentrations of TF

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**Figure 2.** TFPI anticoagulant activity during thrombin generation and its dependence of protein S. Thrombin generation in TFPI-depleted plasma was initiated with 1.4 pmol/L TF, 10 pmol/L phospholipid vesicles, and 16 mmol/L CaCl\(_2\) (final concentrations) in the presence of 30 \(\mu\)g/mL corn trypsin inhibitor (CTI, Hematologic Technologies) and followed with the fluorogenic substrate Z-Gly-Gly-Arg-AMC.HCl.\(^\text{11}\) A, Reconstitution of full-length TFPI in TFPI-depleted plasma at indicated final concentrations (0 to 3.2 nmol/L). B, Reconstitution of full-length TFPI in TFPI-depleted plasma to which inhibitory antiprotein S antibodies (Zebra Bioscience, Enschede) were added.
TFPI-depleted plasma reconstituted with (●) 350 nmol/L protein S, or (○) 350 nmol/L protein S and 0.5 nmol/L full-length TFPI. Averages of duplicate measurements are shown. Adapted with permission from Hackeng et al.27

TFPI: FXa Inhibitor, TF-FVIIa Inhibitor, or Both?

TFPI is an inhibitor of coagulation that has 2 targets, FXa and TF-FVIIa, and theoretically it is possible that the TFPI/protein S system downregulates thrombin formation and coagulation at 2 levels (ie, FXa and TF-FVIIa). It is generally accepted that inhibition of TF-FVIIa by TFPI is physiologically important, whereas virtually no information is available as to whether inhibition of FXa by TFPI also contributes to the downregulation of thrombin formation by TFPI. Although incorporation of FXa into a phospholipid-bound FX-FVa (prothrombinase) complex had no effect on the inhibition of FXa by TFPI in the absence of prothrombin,33 TFPI appeared to be a poor inhibitor of the prothrombinase complex in the presence of physiological concentrations prothrombin.45 As such, TFPI is not different from other anticoagulant proteins (antithrombin and APC) whose anticoagulant activities are also greatly reduced when their targets (FXa and FVa) are incorporated into the prothrombinase complex.46,47 However, TFPI appeared to be an effective inhibitor of prothrombin activation in model systems when FVa was substituted by FV45 (ie, when FV had to be activated by FXa or traces of thrombin before the onset of prothrombin activation).

Of course a possible contribution of direct inhibition of FXa by TFPI to the downregulation of thrombin formation should be put in the perspective of other FXa inhibitors in plasma (eg, antithrombin). At the plasma antithrombin concentration of 2.5 μmol/L,18 and a rate constant of FXa inhibition22 of 3.53×10^(-5) M^(-1)sec^(-1), antithrombin requires ≈1.3 minutes (t1/2) to inhibit 50% of the FXa formed. Using rate constants and Ks reported by Hackeng et al27 it can be calculated that at the plasma-free TFPI concentration (0.25 nmol/L) the t1/2 for inhibition of FXa by TFPI drops from ≈5
Inhibition by TFPI. This implies that the stimulatory effect of protein S on negatively charged phospholipids surfaces is important for the inhibition of FXa that escapes FVIIa-TF-Xa complex. Early thrombin generation will activate platelets, activate factors V and VII, and in addition activates FXI from the propagation loop. (2) FXa can activate FII. FXa and cofactor FVIIa assemble on negatively charged phospholipids, and activate additional FX for participation in prothrombin activation and thus form sufficient thrombin to generate fibrin polymers to stabilize the platelet plug. Thrombin generation is tightly regulated by 2 protein S–dependent feedback mechanisms (3, 4) and protease inhibitors that scavenge coagulation enzymes at large (5). The TFPI/protein S pathway inactivates FXa and subsequently shuts down FX/FVIIa. (3) Thrombomodulin-bound thrombin activates protein C, after which the APC/protein S pathway inactivates FVa and FVIIa (4), both protein S-dependent pathways together limiting generation of the components of the prothrombinase complex, FXa and FVa.

Conclusions

Novel insights in protein S anticoagulant activity resulted in the perception of a protein S–dependent double-barrel negative feedback system that is constitutively active and that is aimed at limitation of prothrombinase activity (Figure 4). On one side protein S stimulates TFPI to inhibit FXa generation by TF/FVIIa, and on the other side protein S enhances the activity of basal levels of APC in FVa inactivation, which in conjunction with other protease inhibitors leads to effective attenuation of coagulation.

Disclosures

None.

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Protein S as Cofactor for TFPI
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