The role of the C-terminal domain in the inhibitory functions of tissue factor pathway inhibitor

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Abstract Tissue factor pathway inhibitor (TFPI) inhibits the activity of coagulation factors VIIa and Xa through Kunitz domains, thereby inhibiting the activity of tissue factor. However, it has been shown that the C-terminal of this inhibitor is essential for the maximal anticoagulant activity of TFPI. We have investigated the endogenous ability of the C-terminal of TFPI to influence coagulation. A synthetic peptide corresponding to residues 254–265 within the C-terminal of TFPI was prepared and shown to be capable of inhibiting tissue factor pathway by preventing the activation of factor VII. Mutational analysis of the peptide revealed the identity of the key lysine residues.

Key words: Tissue factor; Tissue factor pathway inhibitor; Inhibition; C-terminal; Kunitz; Lysine; Arginine

1. Introduction

The exposure of tissue factor upon injury to the endothelium initiates the extrinsic pathway of coagulation [1,2]. The ability of the tissue factor pathway inhibitor (TFPI) to inhibit the extrinsic pathway of coagulation has been clearly demonstrated [3,4]. TFPI contains three tandem Kunitz domains, the first two inhibiting factors VIIa and Xa, respectively [4]. Sequencing of the TFPI cDNA revealed that TFPI encompasses a highly basic C-terminal domain containing repeated lysine and arginine residues [5]. The C-terminal peptide of TFPI has been shown to be involved in the optimal activity of TFPI [6,7]. Furthermore, it has been demonstrated that, while the full-length TFPI and C-terminal truncated TFPI have similar potencies to inhibit factor Xa, the quaternary complex tissue factor-factor VIIa-TFPI-factor Xa is much more stable in the presence of full-length TFPI [8]. This indicated further that interactions occur between the C-terminal of TFPI and tissue factor-factor VIIa complex. In our previous work, we demonstrated that the function of full-length TFPI which associates with LDL is suppressed upon induction of oxidation [9]. Conversely, C-terminal truncated TFPI was unaffected by the treatment. Moreover, the non-specific chemical modification of arginine and particularly lysine residue drastically reduced the effectiveness of TFPI [9]. We have now demonstrated a novel anticoagulant activity of a peptide (residues 254–265) within the C-terminal of TFPI, which inhibits the tissue factor and prothrombinase complex independently of the Kunitz domains, and have identified the key residues required for this activity which include basic amino acids. We propose, therefore, an explanation for the disparity in the abilities of full-length and C-terminal truncated TFPI to inhibit the extrinsic pathway of coagulation.

2. Material and methods

2.1. Preparation of synthetic peptide, mutants and controls

Solid phase peptide synthesis was carried out according to established methods [10], but using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (PerSeptive Biosystems, Herts, UK) for superior coupling and to minimise racemisation [11]. In addition, peptides were synthesised to order (Genosys Biotechnologies, Cambridge, UK). The peptides included the wild-type (WT) (KTKRKRKKQRVK) and mutants containing Ala substitutions in each of the 12 positions (N- to C-terminal), in turn (Mut 1–12). The synthesised peptides were analysed for molecular weight and purity by mass spectroscopy and high performance liquid chromatography. Due to the proportion of positively charged amino acids (nine out of 12) within the sequence, no attempt was made to produce reverse or scrambled variants of the peptide. Instead, control peptides were prepared with high proportions of basic amino acids (SHRHKNKKSAQK and KAHSKTRSKTHNQK) or purchased which were poly-L-lysine, poly-L-arginine, control peptide (TRLTRKRGLKLATAL) (Sigma Chemical Company, Poole, UK).

2.2. Measurement of tissue factor activity

Tissue factor activity was measured using both the one-stage prothrombin time assay and an amidolytic assay as described previously [12] and the percentage inhibition calculated. In some experiments, factors VII and X were pre-incubated with the samples prior to assay. The two-stage amidolytic assay was carried out according to published procedures [13] using *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide obtained from (Sigma Chemical Company, Poole, UK). Also, in a variation factor Xa activity was measured using *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide. To test the effects of the various peptides, they were pre-incubated, over a range of concentrations (0–500 nM) with tissue factor for 0–40 min before application of either method. The tissue factor activity was then calculated from a standard curve and the percentage inhibition due to the peptide being tested was calculated. Finally, a similar experiment was carried out, but the peptide (100, 150 and 200 nM final concentration) was incubated with PMA-treated THP-1 cells exhibiting activity [14] equivalent to 50 nM recombinant tissue factor.

2.3. Measurement of thrombin, factor Xa, factor V and VII activity/activation

The ability of the $TFPI_{254–265}$ peptide (0–500 nM) or control peptides to influence the enzymatic and procoagulant activity of thrombin, factor X (Sigma Chemical Company, Poole, UK) and V (Diagnostic Reagents Oxon, UK) were assessed as described previously [12]. Furthermore, the effect of the $TFPI_{254–265}$ peptide on factor VIIa

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activity and factor VII (Sigma Chemical Company, Poole, UK) activation was investigated according to the procedure previously described [12].

2.4. Statistical analysis

Data were expressed as means and S.E.M. for at least three experiments, unless otherwise stated. The significance values were obtained using unpaired Student's *t*-test. Except where stated, the data were comparable for both the one-stage prothrombin time assay and two-stage amidolytic assay.

3. Results

3.1. Effects of $TFPI_{254–265}$ peptide on tissue factor activity

Incubation of $TFPI_{254–265}$ peptide (0–500 nM) with tissue factor (200 nM) at 37°C produced a concentration-dependent inhibition of the procoagulant activity (Fig. 1). The control peptides, poly-lysine, poly-arginine, a synthetic basic peptide (TRLTRKRGLKLATAL) and two other recombinant basic peptides (TTSHRHKNKKSAQK and KAHSKTRSKTHNQK) showed no inhibitory activity against tissue factor, indicating that the effects of $TFPI_{254–265}$ peptide are sequence specific and not a general effect of basic peptides. Time-course studies using 500 nM $TFPI_{254–265}$ peptide indicated that an inhibition of $>95\%$ was attained following 15 min pre-incubation (Fig. 1). Moreover, the presence of either factor VIIa, Xa or $CaCl_2$ was not a pre-requisite to the inhibitory action by $TFPI_{254–265}$ peptide. When used against activated cells, the peptide exhibited a comparable concentration-dependent inhibition of the procoagulant activity (Fig. 2).

3.2. Mutational analysis of $TFPI_{254–265}$ peptide

The alanine substitution of all 12 amino acids in $TFPI_{254–265}$ peptide revealed a number of key inhibitory amino acids within the peptide sequence, as well as identifying the non-participating amino acids (Table 1). Mutation of lysine residues at positions 3, 5, 7, 8 and 12 (representing positions 256, 258, 260, 261 and 265 in TFPI) or the glutamine at position 9 (262 in TFPI) reduced the inhibitory potential of the peptide by over 65%, with the lysines 7 and 8 being the most important residues.

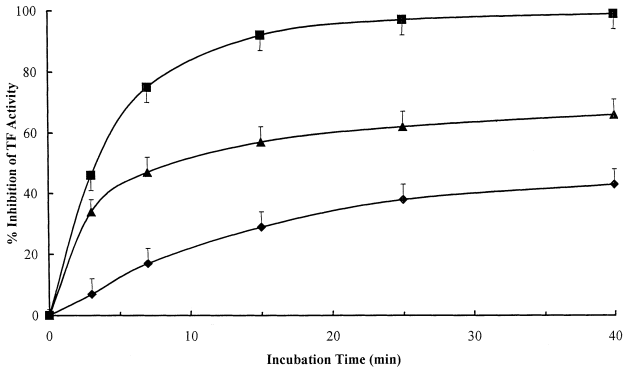

Fig. 1. Time-course assay of the inhibition of the procoagulant activity of recombinant tissue factor by $TFPI_{254–265}$ peptide. Three 1 ml samples of $TFPI_{254–265}$ peptide (100 (♦), 200 (▲) and 500 (■) nM) were incubated with recombinant human tissue factor (200 nM) and samples were removed at various times up to 40 min and assayed. Control peptides were also included in the assay (not shown). The percentage inhibition of tissue factor activity was calculated from a standard curve against the control sample. The data were obtained from four independent experiments each carried out in duplicate (mean ± S.E.M.).

3.3. Measurement of influence of $TFPI_{254–265}$ peptide on thrombin, factor Xa and factor V activity

Pre-incubation of the $TFPI_{254–265}$ peptide (500 nM) with thrombin did not affect either the clotting ability or the amidolytic proteolytic activity of the latter (100 nM) (not shown). The $TFPI_{254–265}$ peptide (500 nM) also did not affect the amidolytic activity of factor Xa (not shown), indicating that it does not interfere with enzymatic action of this clotting factor. However, some inhibition of coagulation was observed upon pre-incubation of $TFPI_{254–265}$ peptide (500 nM) with either factor Xa (100 nM) or factor V (1 μM) (Fig. 3), indicating some interference with prothrombinase complex assembly. This was also repeated with two lower concentrations of $TFPI_{254–265}$ peptide (100 and 200 nM) and inhibition was also observed. Hence, the ability of $TFPI_{254–265}$ peptide to inhibit the clotting activity, but not the enzymatic activity of factor Xa, suggests that the peptide interferes with the interactions

Table 1
The influence of alanine mutations of $TFPI_{254–265}$ peptide

Peptide	Sequence	Procoagulant activity	% Inhibition
Control	–	50 U/ml ± 5	0%
WT	KTKRKRKKQRVK	0.5 U/ml ± 3*	99%
M1	**A**TKRKRKKQRVK	7.8 U/ml ± 4*‡	84%
M2	K**A**KRKRKKQRVK	2.5 U/ml ± 4*	95%
M3	KT**A**RKRKKQRVK	36 U/ml ± 5†	28%
M4	KTK**A**KRKKQRVK	2.5 U/ml ± 2*	95%
M5	KTKR**A**RKKQRVK	32.5 U/ml ± 4†	35%
M6	KTKRK**A**KKQRVK	7.5 U/ml ± 3*‡	85%
M7	KTKRKR**A**KQRVK	40 U/ml ± 5†	20%
M8	KTKRKRK**A**QRVK	36.5 U/ml ± 5†	27%
M9	KTKRKRKK**A**RVK	32 U/ml ± 2†	36%
M10	KTKRKRKKQ**A**VK	10 U/ml ± 4*‡	80%
M11	KTKRKRKKQR**A**K	0.3 U/ml ± 2*	99%
M12	KTKRKRKKQRV**A**	35 U/ml ± 5†	30%

Samples of $TFPI_{254–265}$ peptide and 12 mutant peptides (500 nM) were incubated with tissue factor (50 nM (50 arbitrary units/ml)) at 37°C for 10 min prior to assaying. The activity was calculated from a tissue factor standard curve. The data were expressed as mean ± S.E.M., obtained from three independent experiments. Statistical analysis carried out by *t*-test. * $P>0.01$ against control (no peptide), † $P>0.01$, ‡ $P>0.05$ against WT $TFPI_{254–265}$ peptide.

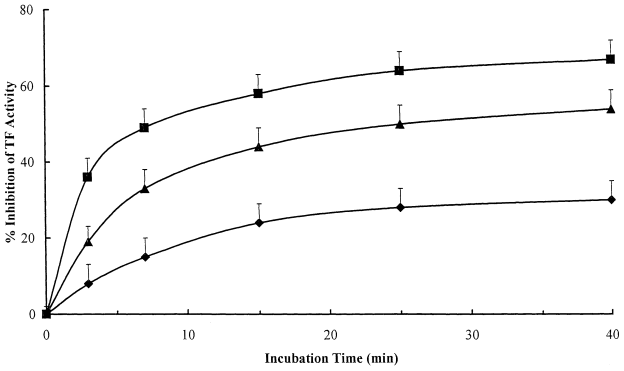

Fig. 2. Time-course assay of the inhibition of the procoagulant activity of PMA-treated THP-1 monocytic cells by EPIC peptide. THP-1 monocytic cells were incubated with 10 nM PMA for 4 h. The cells were then centrifuged and resuspended to 10^7 cells/ml in phosphate-buffered saline. Two-hundred μl aliquots were incubated with 50 μl $TFPI_{254–265}$ peptide (100 (♦), 150 (▲) and 200 (■) nM final concentration) at 37°C. A control peptide was also included in the assay (not shown). The residual tissue factor activity was monitored over 40. The percentage inhibition of tissue factor activity was calculated against the control sample. The data were obtained from three independent experiments (mean ± S.E.M.).

involved in prothrombinase complex assembly rather than being a direct inhibitor of factor Xa.

3.4. Measurement of the effect of $TFPI_{254–265}$ peptide on the activation and activity of factor VII

Pre-incubation of $TFPI_{254–265}$ peptide (500 nM) with factor VII (50 nM), prior to addition to tissue factor (200 nM) and factor VII-deficient plasma, did not change the activity of the enzyme (inhibition = 5% ± 7). In order to assess whether the action of $TFPI_{254–265}$ peptide was directed to the proteolytic activity of factor VIIa/tissue factor or the activation of factor VII by interaction with tissue factor, tissue factor (200 nM) and factor VII (200 nM) were incubated for 10 min in the presence of 25 mM $CaCl_2$, prior to addition of $TFPI_{254–265}$ peptide (500 nM) followed by a further 10 min incubation. In a second experiment, tissue factor, factor VII and factor Xa (100 nM) were pre-incubated for 10 min in the presence of 25 mM $CaCl_2$, prior to addition of $TFPI_{254–265}$ peptide and a further 10 min incubation. The samples were then assayed by addition to factor VII-deficient plasma and measurement of the clotting time. $TFPI_{254–265}$ peptide did not influence the activity of factor VIIa (inhibition = 10% ± 8). In contrast, the peptide was able to inhibit the activation of the pro-enzyme (inhibition = 70% ± 8). This indicates the ability of the peptide to inhibit the activation of factor VII, but not the activity of pre-activated factor VIIa.

4. Discussion

There are two forms of TFPI detected in vivo [6,7], which are identical in sequence with the exception that the lighter truncated form does not possess the highly basic C-terminal. The C-terminal truncated TFPI has a lower inhibitory potential than full-length TFPI, indicating that the C-terminal peptide has a functional role against tissue factor activity [6,7]. Furthermore, although the two forms of TFPI have similar abilities to inhibit factor Xa, the formation of the quaternary complex tissue factor-factor VIIa-TFPI-factor Xa occurs more readily with the full-length TFPI [8]. The reason for the presence of the two forms of circulating TFPI with different inhibitory potentials is as yet unknown, but may be significant in the proper control of haemostasis. Here, we suggest a possible explanation for the disparity in the level and stability of inhibition observed between full-length and truncated TFPI.

It has been shown previously that full-length TFPI may associate with LDL with a resultant reduction in the overall activity of the complex towards tissue factor [9,15]. Recently, Bridey et al. recorded a 2-fold enhancement of the specific activity of LDL-bound TFPI as compared to free TFPI, although the nature of TFPI in the fractions was not determined [16]. However, since the concentration of plasma apolipoprotein (apo) B-100 is approximately 50 times that of TFPI, it is also possible that the tissue factor inhibition by the LDL/LDL-TFPI may arise from the TFPI-free LDL only. Indeed, a higher specific activity of factor VIIa (factor VIIa activity/factor VIIa antigen) (2.5–3-fold) has been observed in patients with a β-lipoproteinemia [17], indicating that LDL markedly attenuates factor VIIa activity. The ability of the receptor binding domain of apo B-100 (residues 3147–3160) to inhibit tissue factor arises from the direct interaction of lysine residues within this domain with negative residues within tissue factor (residues 58–66) was shown in earlier work from this laboratory [12,18–21]. In comparison to the apo B-1000-derived peptide [12], the ability of $TFPI_{254–265}$ peptide to inhibit tissue factor was similar in magnitude. Alignment of the two sequences revealed a number of identical (bold) and similar (underlined) amino acids.

Position	1	2	3	4	5	6	7	8	9	10	11	12	13
TFPI (254-265)	**Lys**	Thr	**Lys**	Arg	Lys	Arg	**Lys**	**Lys**	Gln	Arg	Val	Lys	
Apo B-100 (3147-3157)			**Lys**	Ala	Gln	Tyr	**Lys**	**Lys**	Asn	Lys	His	Arg	His

In the case of $TFPI_{254–265}$ peptide, we have now performed a more extensive mutational analysis than was performed with the apo B-100-derived peptide. Nevertheless it is still possible to compare the effects of the mutations in some of the key sites, especially those at positions 7, 8 and 9 which are clearly important in the inhibition of both $TFPI_{254–265}$ peptide and apo B-100-derived peptides, although minor differences occur. Of particular note are the alanine substitutions of lysines 3151 and 3152 (positions 7 and 8 above) and asparagine 3153 (in position 9) within the apo B-100-derived peptide, which results in large reduction of the inhibitory function of this peptide [12]. Equally, the residue in position 10 was shown not to be of significance in both cases. The lysines at positions 3, 5 and 12 in $TFPI_{254–265}$ peptide were also found to be essential, but the importance of the equivalent amino acids in apo B-100-derived peptide has yet to be established. We opted to carry out mutational analysis of these amino acids in turn, within the C-terminal peptide rather than within full-length TFPI. Although the mutation of a single amino acid within the C-terminal affects the inhibitory potential of the TFPI, this effect would have been less evident since the intact Kunitz domains were still active. Therefore, the effect of the C-terminal domain on the TFPI would only become easily detectable when the whole C-terminal is missing, as in the natural truncated form [6,7].

Aspartate 58 within the sequence of tissue factor is involved in the binding [22,23] and activation of factor VII [24]. If this

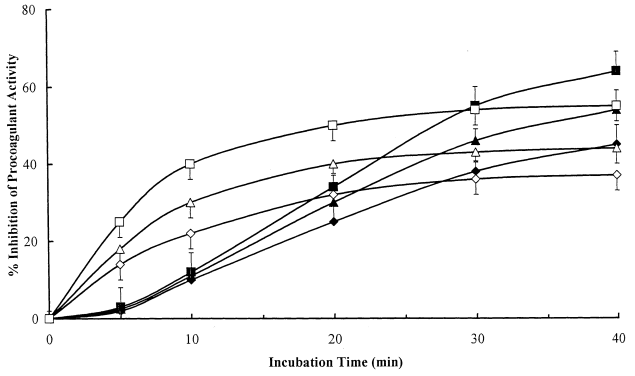

Fig. 3. Time-course assay of the inhibition of the procoagulant activity of factor Xa by $TFPI_{254–265}$ peptide. (a) One ml samples containing $TFPI_{254–265}$ peptide (500 (■), 200 (▲) and 100 (♦) nM) were mixed with factor Xa (100 nM). One-hundred μl of the samples was removed immediately and assayed for factor Xa activity by addition to factor Xa-deficient plasma in the presence of $CaCl_2$ (25 mM) and measuring the clotting time. The rest of the sample was incubated at 37°C and further 100 μl samples were removed at intervals up to 40 min and measured as before. The activity was measured against a standard curve and the percentage inhibition calculated against the initial factor Xa activity. (b) One ml samples containing $TFPI_{254–265}$ peptide (500 (□), 200 (△) and 100 (◇) nM) were mixed with factor V (1 μM). One-hundred μl of each sample was removed immediately and assayed for factor Xa activity by addition to factor V-deficient plasma, pre-activated by incubation (2 min) with tissue factor (200 nM) in the presence of $CaCl_2$ (25 mM) and measurement of clotting time. The rest of the sample was incubated at 37°C and further 100 μl samples were removed at intervals up to 40 min and measured as before. The activity was measured against a standard curve and the percentage inhibition calculated against the initial factor V activity. The data were obtained from three independent experiments (mean ± S.E.M.).

domain is masked by these peptides, it would interrupt the correct factor VII-tissue factor complex formation and subsequent factor VII activation. Therefore, full-length TFPI has a dual action in inhibiting tissue factor directly, as well as the activities of factor Xa and VIIa.

In conclusion, the novel anticoagulant property of a peptide rich in basic amino acids (residues 254–265) within the C-terminal of TFPI has been demonstrated acting on tissue factor and prothrombinase complex independently of the Kunitz domains. An explanation is proposed for the disparity in the abilities of full-length and C-terminal truncated TFPI to inhibit the extrinsic pathway of coagulation.

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