Initiation of the Tissue Factor Pathway of Coagulation in the Presence of Heparin: Control by Antithrombin III and Tissue Factor Pathway Inhibitor

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Activation of factor X by both the unactivated tissue factor-factor VII complex (TF:VII) and the activated tissue factor-factor VIIa complex (TF:VIIa) has been studied in the presence of tissue factor pathway inhibitor (TFPI), antithrombin III (ATIII), and heparin. At near-plasma concentrations of TFPI, ATIII, and factor X, factor X activation that occurs in response to TF:VII is essentially abolished in the presence of heparin (0.5 μmol/L). This effect requires both inhibitors, acting on different targets: (1) ATIII, which in the presence of heparin blocks the activation of TF:VII, and (2) TFPI, which inhibits the TF:VIIa that is generated. In the absence of ATIII, TFPI alone with or without heparin reduces but does not abolish factor X activation. Conversely, in the absence of TFPI, ATIII + heparin reduces but does not abolish TF:VIIa generation and allows continuing activation of factor X. These results indicate that when the unactivated TF:VII complex is the initiating stimulus, heparin-dependent reduction in the rate and extent of factor X activation requires both ATIII and TFPI. In contrast, if TF:VIIa is used to initiate activation, only TFPI is involved in its regulation.

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the level of any contaminating factor Xa and factor VIIa, the supplied protein was diluted in 4 vol Tris-buffered saline, pH 7.5, and then treated with 0.5 μmol/L DnsEGRck and 50 μmol/L FFRck for 30 minutes at 37°C, followed by exhaustive dialysis (2 × 200 vol) against 0.15 mol/L NaCl/5 mmol/L MES/NaOH, pH 6.0. The resulting contamination with factor VIIa, estimated from parameters of autolytic activation in the presence of tissue factor, is less than 1%.

Recombinant human factor VIIa was a generous gift from Novo Nordisk (Bagvaerd, Denmark). Recombinant TFPI was produced in an Escherichia coli expression system. The TFPI was greater than 90% active, as determined by titration against a pure standard sample of factor Xa. Recombinant full-length human tissue factor was generously provided by Dr. William Konigsberg of Yale University (New Haven, CT). It was reticulated with 30% PS/70% PC (PS:PC) in the presence of 0.5% octylglucoside, and exhaustively dialyzed against HEPES-buffered saline (0.1 mol/L NaCl in 50 mmol/L HEPES-NaOH, pH 7.5) to remove detergent and form vesicles. Lipid to protein ratios were chosen to provide a constant 25-μmol/L concentration of phospholipid in all experiments. At the tissue factor concentration used in the majority of this study (0.5 mmol/L), the lipid to protein ratio is 5 × 10⁻³.

Radioiodination of factor X. Factor X was labeled with Na¹²⁵I by a mild method that uses H₂O₂ and lactoperoxidase in solution. The lactoperoxidase, itself, which remains (albeit inactive) in the labeled preparation, amounts to less than 2% of the total protein. It is also of higher molecular weight than either chain of factor X, and can be ignored. Different batches of labeled factor X varied from 5,000 to 8,000 cpm/μmol. All were fully active by bioassay, but this is unsurprising, since the molar incorporation of iodine was uniformly less than 0.1 g atom iodine/mmol protein. Validation of the kinetics of activation of labeled material are described later.

Measurement of [¹²⁵I]-factor X activation. Incubations were set up to contain 100 mmol/L unlabeled factor X, 1 to 2 mmol/L [¹²⁵I]-factor X, and other additions as described later. Samples from incubations were taken at specified times and added to an equal volume of a solution containing 10 mmol/L EDTA/5%/2-mercaptoethanol/0.025% bromophenol blue/15% glycerol/2%/sodium dodecyl sulfate/80 mmol/L imidazole/HCl, pH 6.4, and heated to 95° to 100°C for 3 minutes. These samples (20 μL, containing ~7 × 10⁴ cpm) were run on discontinuous polyacrylamide gels in dodecyl sulfate (stacking gel of 5% acrylamide in imidazole/HCl, pH 6.8, separating gel of 12.5% acrylamide in Tris/HCl, pH 8.5).

After electrophoresis, the gels were fixed in 25% isopropanol/10% acetic acid, dried, and exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) for 18 to 24 hours. Initial tests of standardized samples of varying radioactive content showed that densitometry of autoradiogram bands did not suffice for linear quantification of radiolabeled protein. Autoradiograms were therefore used solely to indicate where the radioactive (factor X) bands were on the dried gel. The localized bands were then cut out and counted in a gamma counter.

Since the light chain (LC) of factor X remains uncleaved during factor X activation, it can be used as an internal standard, i.e., it can be used to correct for variations in the volume of samples applied to the gel. Each gel lane (sample) was therefore cut (using the autoradiogram as a guide) to yield two samples ("bands") for determination of radioactive content: (1) the factor X heavy chain (HC), which disappears upon and is a measure of activation, and (2) the stable, uncleaved LC. The counts per minute of HC samples was divided by the counts per minute of LC samples to yield an HC/LC ratio.

Control activations of factor X by 0.25 mmol/L TF:VIIa in the absence of any inhibitors showed that complete activation corresponded—with this particular method of radioiodination—with a mean change in the HC/LC ratio from 1.75 to 0.18. The nonzero value of the ratio in completely activated samples reflects both background radioactivity in the excised gel samples and the existence of a small proportion (7%) of labeled factor X that is resistant to activation. Knowing the change in the HC/LC ratio for complete activation and knowing the total concentration of (unlabeled) factor X, we could convert measured HC/LC ratios in experimental samples to nanomolar concentrations of factor X. It should be noted that the method is prone to sporadic inaccuracies, chiefly caused by the occasional broadening of bands due to inconsistencies in the gel, and hence incomplete recovery of the pertinent chain for counting. This appears as occasional noise in the data.

Experimental conditions. The factor X level throughout was 100 nmol/L. Factor VIIa was held in excess over tissue factor, at 1 nmol/L. Tissue factor was present at 0.5 mmol/L in PS:PC vesicles. If we assume that only half the protein inserts in the vesicle membrane right-side out, the effective concentration is 0.25 nmol/L. Given the high affinity of the TF:VII interaction (Kᵦ = 7 pmol/L), we may assume the concentration of TF:VII or TF:VIIa in these experiments to be 0.25 nmol/L.

Except in initial experiments involving TFPI inhibition of TF:VIIa in the absence of ATIII (Fig 2), a single TFPI concentration was chosen to produce a reliably measurable reduction in factor X activation under the conditions described. A partial rather than total reduction in factor X activation was needed to allow clear demonstration of a further reduction in the presence of heparin. A concentration of 1 nmol/L TFPI was used, and in the absence of ATIII this halts factor X activation after consumption of about 70% of factor X. ATIII concentration was 5 μmol/L close to its plasma level. Heparin was used at a concentration of 0.5 μmol/L.

RESULTS

Radiolabeled factor X as an indicator of factor X activation. Studies of factor X activation in the presence of factor Xa inhibitors require measurement of factor X activation by methods that do not rely on the enzymic activity of factor X. The radioiodinated factor X was labeled to specific incorporations of less than 0.1 g atom I/mmol, and it was necessary to confirm that the disappearance of radiolabeled factor X correlates with the activation of bulk unlabeled factor X. Figure 1 compares the results—factor X activation and factor Xa generation—in incubations containing radiolabeled factor X (~1 nmol/L), unlabeled factor X (100 nmol/L), and either TF:VII or TF:VIIa. Note that the disappearance of radiolabeled factor X involves a later slower phase that is not discernible in the time courses of factor Xa generation. We assume this is caused by a fraction of the radiolabeled factor X, amounting to 20% to 25% of the total, that is activated more slowly by TF:VIIa. However, although the agreement in the kinetics is not perfect, the data (Fig 1) show that radioiodinated factor X is a satisfactory substrate for TF:VII(a) for the purposes of this study.

Figure 1 also shows the near-identical time courses of factor X activation by TF:VII and TF:VIIa, demonstrating the rapidity of activation of TF:VII. From a comparison of the initial kinetics of factor X activation by the two complexes, we can estimate that TF:VII activation is complete in this system in less than 15 seconds.

Effect of TFPI and heparin in the presence of ATIII. We first studied the activation of factor X by TF:VIIa in the presence of TFPI from 0 to 5 nmol/L in the absence of other factor Xa inhibitors, using a discontinuous chromogenic
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The absence of heparin, and the final extents of activation are similar. However, at 5 nmol/L TFPI, heparin reduces the rate and extent of activation threefold more than TFPI alone. The relation of these results to a previous report of the kinetics of heparin action on TFPI is considered later. Figure 2 also demonstrates that heparin alone, in the absence of other inhibitors, has no significant effect on the activation of factor X by TF:VIIa.

Effect of ATIII and heparin in the absence of TFPI. Figure 3 shows the effect of heparin on factor X activation by TF:VIIa or TF:VII in the presence of ATIII. TFPI was absent in these experiments. The rate and extent of factor X activation by TF:VIIa are almost unaffected by heparin. This result confirms that ATIII is not a major control of TF:VIIa even in the presence of heparin. Unactivated TF:VII was also used, meaning that TF:VIIa generation was required for factor X activation. In this situation, heparin reduces the rate and extent of factor X activation by at least 80%. Since the direct effect of ATIII + heparin on TF:VIIa is relatively small, we may conclude that the major effect of heparin (in the presence of ATIII) is to decrease activation of TF:VII by factor Xa. The slow activation of factor X that remains is likely a result of autolytic activation of TF:VII by TF:VIIa.

Effect of ATIII and heparin in the presence of TFPI. To this point, it is clear that TFPI and ATIII both have significant effects in the presence of heparin, but their target enzymes are apparently different. We show the effect of adding both inhibitors together, with and without heparin (Fig 4). In comparing the data of Fig 4A and B with Fig 3A and B, it is clear that in the presence of TFPI, factor X activation shuts down earlier and more completely than in its absence, consistent with the rapid inhibition of TF:VIIa by the absence of heparin.
Fig 3. Effect of heparin on factor X activation by TF:VII(a) in the presence of ATIII. Factor X 100 nmol/L + [125I]-factor X 1.2 nmol/L + ATIII 5 μmol/L were added to TF 0.25 nmol/L in the presence (○) or absence (□) of heparin 0.5 μmol/L plus Ca²⁺ 5 mmol/L. Factor X activation was started with 1 nmol/L factor Vlla (A) or factor VII (B) and was followed by measurement of HC/LC [125I] distribution.

TFPI:Xa complex. Where TF:VIIa is provided and no activation of TF:VII is required, factor X activation ceases in 1 minute in the presence of heparin and in about 2.5 minutes in its absence (Fig 4A). Since the results of Fig 3 rule out a large effect of ATIII on TF:VIIa under these conditions, the larger part of the heparin effect probably results from the direct acceleration of the interaction of TFPI with factor Xa (Fig 2).²⁹,³⁰

Where TF:VII activation is required, the effect of heparin is much more pronounced, producing a near-total (>98%) block of factor X activation (Fig 4B). We may conclude that although ATIII + heparin does not totally abolish TF:VII activation (Fig 3B), the small amount of TF:VIIa that is formed is subject to rapid and complete inhibition in the presence of TFPI.

Effect of DnsEGRck and heparin in the presence of TFPI. These results suggested that when ATIII and TFPI are both present with TF:VII, heparin exerts its effect via two routes: a large effect via ATIII on the feedback activation of TF:VIIa, and a smaller direct effect on TFPI. However, the data of Fig 4 do not allow any meaningful assignment of the relative effects of heparin on the two routes of control. To delineate the heparin dependence of TFPI, while maintaining factor Xa inhibition at a rate unaffected by heparin, experiments were performed in the presence of TFPI but using a factor Xa inhibitor that is essentially unaffected by heparin, DnsEGRck. Under these conditions, any observed heparin-dependent effects on factor X activation must be TFPI-dependent effects.

Control experiments (not shown) showed that 0.3 μmol/L DnsEGRck inhibits factor Xa at approximately the same rate as the 5 μmol/L ATIII used in prior experiments (0.45 ± 0.50 min⁻¹, respectively) and that inhibition by DnsEGRck is only slightly accelerated by heparin (0.62 ± 0.45 min⁻¹). Further controls showed that 0.3 μmol/L DnsEGRck does not significantly inhibit TF:VIIa, either with or without heparin (k = 0.016 and 0.090 min⁻¹, respectively).

Figure 5 shows the activation of factor X in systems containing TFPI and DnsEGRck, with and without heparin. Factor X activation by TF:VIIa is about 30% slower in the presence of heparin and somewhat less complete, reflecting

Fig 4. Effect of heparin on factor X activation by TF:VIIa in the presence of TFPI and ATIII. Factor X 100 nmol/L + [125I]-factor X 1.2 nmol/L + ATIII 5 μmol/L + TFPI 1 nmol/L were added to TF 0.25 nmol/L in the presence (○) or absence (□) of heparin 0.5 μmol/L plus Ca²⁺ 5 mmol/L. Factor X activation was started with 1 nmol/L factor VIIa (A) or factor VII (B) and was followed by measurement of HC/LC [125I] distribution.
the direct effect of heparin on TFPI (Fig 5A). The rate and extent of factor X activation by TF:VII (Fig 5B) are more similar still. By comparison with Fig 4, the results of Fig 5 confirm convincingly that although TFPI is indeed required for the observed heparin dependence in the control of factor X activation by TF:VII, the major target of heparin action under these conditions is inhibition of factor Xa by ATIII.

DISCUSSION

It is generally accepted that tissue factor is the normal initiator of coagulation, the initial event being its combination with factor VII to form the TF:VII complex. The complex has little or no enzymic activity. In plasma, there is also a low idling level of the enzyme factor VIIa, typically less than 0.5% of the factor VII level, but no significant generation of factor Xa can be realized until the bulk TF:VII complex is activated. Of the several activators of TF:VII known, two would seem significant in physiologic terms: the TF:VIIa complex itself and factor Xa. Of these, the latter is much more potent, and is probably largely responsible for

the bulk activation of TF:VII. However, in the initial stages, before significant factor Xa is generated, TF:VIIa itself may have an important role. Two inhibitors are also involved that are both subject to control by heparin, and they act together to control factor X activation.

ATIII. At its plasma concentration, ATIII is an insignificant inhibitor of TF:VIIa and a moderate inhibitor of factor Xa. Heparin accelerates both reactions, but the resulting rates are very different: ATIII in the presence of heparin inhibits TF:VIIa at a second-order rate of 0.34 μmol⁻¹·min⁻¹·L⁻¹ (the concentration referring to the fully saturated ATIII:heparin complex).²⁶ In agreement with these data, in the present study using 0.5 μmol/L heparin and 5 μmol/L ATIII, we obtained a rate of 0.17 min⁻¹. In contrast, the rate of inhibition of factor Xa is at least 50-fold higher, exceeding 10 min⁻¹ under the same conditions. As others have noted,²³ given these relative rates, it is not surprising that even in the presence of heparin, inhibition of factor Xa by ATIII plays a minor role in the regulation of factor X activation by TF:VIIa.

In the current study, we have shown that despite its unimportance in the direct control of TF:VIIa action on factor X, ATIII in the presence of heparin plays a crucial role in regulating initiation of coagulation by TF:VII. The reason is the requirement for feedback activation of TF:VII. The results shown in Fig 3 comparing factor X activation by TF:VII and TF:VIIa show that heparin has a large effect in slowing factor X activation by TF:VII, and confirm that this effect is mainly caused by heparin-dependent acceleration of factor Xa inhibition by ATIII. The evidence of Figs 3 and 4 thus shows that for significant control of factor X generation by heparin, ATIII is critical—and it is clear that heparin acts to block feedback activation of TF:VII. Furthermore, the experiments of Fig 5, using a non-heparin-dependent inhibitor of factor Xa, demonstrate convincingly that this heparin effect requires ATIII.

TF:VIIa complex. The foregoing assumes that in the experimental design, in which factor VIIa is in excess over TF, any contribution of free factor VIIa to the results is negligible. The rate of factor X activation by factor VIIa in the absence of tissue factor is certainly negligible at the 1-nmol/L factor VII(a) concentration used.²⁴ In addition, it is known that free factor VIIa is not subject to significant inhibition by ATIII + heparin.²⁶,²⁷ However, as Rao et al have reported, the dissociation rate of VIIa:ATIII from cell-bound TF is higher than that of factor VIIa alone (0.07 ν 0.023 min⁻¹). If the same is true of the TF:VIIa complex on reconstituted vesicles, it is thus feasible that a slow pseudo-catalytic turnover occurs of the following form: TF:VIIa + ATIII → TF:VIIa:ATIII → TF + VIIa:ATIII. However, given the dissociation rates and the threefold excess of factor VIIa over TF in the present study, such turnover is unlikely to affect the conclusions concerning the role of ATIII in inhibiting TF:VIIa.

TFPI. In addition to ATIII + heparin, control of factor X activation requires TFPI: in the absence of TFPI, the contribution of ATIII + heparin to the inhibition of TF:VIIa or the control of factor X activation is relatively small. Although clarifying the TFPI and ATIII requirements, the pres-
ent study raises interesting questions about the effect of heparin on TFPI. Huang et al. have reported a significant effect of heparin on the affinity of TFPI for factor Xa, and we recently showed that the initial rapid reaction of TFPI with factor Xa is accelerated approximately twofold by saturating levels of heparin, for TFPI concentrations in the subnanomolar range. However, the present results suggest that the latter data cannot be extrapolated to higher TFPI levels: Fig 2 shows that in terms of the overall kinetics of factor X activation by TF:VIIa, the relative effect of heparin (the fractional reduction in rate and extent of factor X activation) increases with increasing TFPI concentration.

**TFPI + ATIII + heparin.** Although details of the interaction of heparin with TFPI and elucidation of its complex concentration dependence must await studies using other techniques, it is nonetheless clear that the heparin-dependent control of TF:VII requires both TFPI and ATIII. In the absence of ATIII, feedback activation of TF:VII by factor Xa can proceed largely unaffected, and TFPI alone (with or without heparin) is incapable of completely blocking the activation of factor X. Conversely, in the absence of TFPI, although ATIII + heparin can substantially abolish TF:VII activation, any small amount of TF:VIIa that is produced is quite stable and can support continuing factor X activation.

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