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Biochimica et Biophysica Acta, 992 (1989) 409–411
Elsevier

BBAGEN 20244

BBA Report

Free factor Xa is on the main pathway of thrombin generation in clotting plasma

H. Coenraad Hemker [1], Jean Choay [2] and Suzette Beguin [1]

[1] *Department of Biochemistry, University of Limburg, Maastricht (The Netherlands) and* [2] *Institut Choay, Paris (France)*

(Received 28 December 1988)

Key words: Factor X; Thrombin generation; Blood coagulation; (Plasma)

The effect of a synthetic pentasaccharide that specifically causes the inactivation of factor Xa on the development of prothrombinase activity in human plasma was monitored using four triggers of coagulation: (a) human brain thromboplastin; (b) contact activation; (c) factor X activating enzyme complex; (d) prothrombin activating enzyme complex. Inhibition was similar with the triggers a, b and c. With prothrombinase (d), the inhibition strongly decreased with increasing amounts of factor Va present. This indicates that only free factor Xa is inhibited. Because both the intrinsic pathway (b) and the extrinsic pathway (a) are inhibited by the pentasaccharide, we conclude that free factor Xa plays a rate-limiting role in the pathways, so that there is no reason to postulate the existance of 'supercomplexes' consisting of factors IXa, VIIIa, X(a), Va and prothrombin adsorbed on the same phospholipid particle (intrinsic system) or factor VII(a), X(a), Va and prothrombin adsorbed on tissue thromboplastin (extrinsic system).

The series of proenzyme–enzyme conversions that leads to prothrombin activation differs according to the trigger used to start the clotting of blood. With an excess of tissue factor (TF) present, a large amount of the factor VII-TF complex forms which activates factor X. Factor Xa then converts prothrombin (factor II) into thrombin (factor IIa). If there is no tissue factor present the blood coagulation system can be activated by glass, kaolin etc. In that case factor XIa will appear, which activates factor IX. Factor IXa then activates factor X which, once activated, activates prothrombin. When small amounts of TF are present, which is probably the more physiological situation, then not only direct activation of factor X by factor VII-TF plays a role but also indirect activation that occurs because factor VII-TF activates factor IX that then activates factor X [1–5].

Neither factor IXa nor factor Xa is an efficient enzyme when acting alone. They both need to be adsorbed onto a phospholipid surface and next to a protein cofactor in order to obtain a physiologically important activity [6–9]. The cofactor for factor IXa is factor VIIIa; that for factor Xa is factor Va. Both these factors arise from their plasmatic precursors forms by the action of thrombin [10].

Both factor IXa and factor Xa act when adsorbed on a phospholipid surface. It therefore is conceivable that the actual formation of thrombin in plasma occurs at that surface in a cluster of factors IXa, VIIIa X(a), Va and II in which factor X first plays the role of substrate to factor IXa and, once activated acts as the enzyme that activates prothrombin. Indeed, it has been suggested that 'the reactions of the procoagulant cascade may involve sequential channeling of reaction products between enzyme complexes on the same cell surface' [11,12]. Alternatively, factor Xa may dissociate from the lipid once it is formed and from free solution form the factor II activating complex by adsorption next to factor Va onto phospholipid. Analogously in the extrinsic system either a 'supercomplex' may form on the lipoprotein TF when not only factor VII(a) adsorbs there, but also factors X(a), Va and prothrombin. Alternatively, TF-VII(a) may be the enzymatic entity that produces free Xa, which then, with factor Va and phospholipid, forms prothrombinase as in the intrinsic system.

Factor Va is known to enhance the binding of factor Xa to phospholipid more than 1000-fold [13,14]. One can therefore suppose that the first of the two mechanisms mentioned above would be operative if factor Va were present in the immediate vicinity of factor X at the moment it was activated. At this moment it is com-

Abbreviation: IF, tissue factor.

Correspondence: H.C. Hemker, Department of Biochemistry, P.O. Box 616, 6200 MD Maastricht, The Netherlands.

pletely unknown which of the two possible mechanisms is operative under physiological conditions.

In order to decide between these alternatives we measured the time-course of the prothrombinase activity in clotting blood under a variety of conditions. The prothrombinase activity as a function of time was obtained from the thrombin generation curve by a recently developed method [15]. As a first step we determined two pseudo-first-order breakdown constants of thrombin in plasma: k_1, which governs the inactivation of thrombin by AT III and minor inhibitors, and k_2, which determines the formation of the α_2 macroglobulin-thrombin complex. Then the time-course of thrombin generation is assessed at 10–30 s intervals by subsampling into a solution of the thrombin-specific chromogenic substrate S2238 (HD-phe-pip-arg-pNA). At every measuring point the velocity of prothrombin conversion, i.e., the prothrombinase activity, is calculated as the sum of the experimental thrombin generation velocity and the breakdown velocity calculated from the thrombin concentration at that moment and the breakdown constants. The complications which arise from the fact that the thrombin-α_2M complex has a persistent amidolytic activity, is accounted for [15].

As a tool in these experiments we used the synthetic pentasaccharide that represents the AT III binding site of heparin [16]. This substance markedly enhances the inactivation of factor Xa by AT III but does not influence the breakdown of thrombin activity [17].

In a first series of experiments we determined the inhibition by pentasaccharide of the peak prothrombinase activity that was elicited by tissue thromboplastin or by contact activation (Fig. 1a and b). The inhibition observed was similar in both cases. We

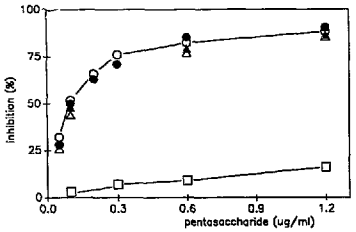

Fig. 1. The inhibition of peak prothrombinase activity by pentasaccharide. Reaction mixture: 240 μl citrated plasma; 60 μl pentasaccharide solution in 50 mM Tris-HCl/100 mM NaCl (pH 7.35); 60 μl trigger solution. At 10 s intervals 10 μl was subsampled into 20 μM S2238 in 50 mM Tris-HCl (pH 7.9)/100 mM NaCl/20 mM EDTA. The thrombin concentration was determined from dO/dt measured at 405 nM. (Full details on the method in Ref. 15. The following triggers were used: (a) ○, human brain thromboplastin diluted 1:40; (b) ●, kaolin, 25 μg/ml and phospholipid 1 μM; (c) △, complete tenase (factor VIIIa, factor IXa, PL); (d) ▲, tenase without factor VIIIa; (e) □, complete prothrombinase (factor Va, factor Xa, PL).

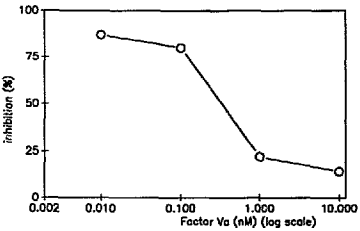

Fig. 2. The factor Va dependency of the inhibition by pentasaccharide of factor Xa-induced prothrombinase activity. Reaction mixture: see Fig. 1. Pentasaccharide concentration: 5 nM.

then prepared a trigger solution of factor-X-activating enzyme (tenase) by adding together (5 nM) factor IXa, (2 nM) factor VIIIa and (12 μM) phospholipid in $CaCl_2$. The phospholipid used was a 20/80% mixture of phosphatidylserine and phosphatidylcholine. For further details and the preparation of the purified factors see legend to Fig. 1 and Ref. 18. At zero time, 60 μl of this mixture was used to trigger thrombin formation, thus shortcutting the factor-IX-activating reactions. The inhibition was similar to that seen in the previous setups (Fig. 1c). Omitting factor VIIIa from the activation mixture did not influence the degree of inhibition by pentasaccharide (Fig. 1c and d). When, however, thrombin formation was triggered by a solution of the prothrombin-activating enzyme (i.e., factor Xa (2 nM), factor Va (12 nM), phospholipid (12 μM), $CaCl_2$ (5 mM), no inhibition was observed (Fig. 1e).

In Fig. 2 it is seen that the inhibition by pentasaccharide of the peak prothrombinase activity in plasma is dependent upon the amount of factor Va present. At high factor Va concentrations the inhibition is negligible, whereas it increases with decreasing factor Va concentration.

As mentioned above, factor Va fosters the binding of factor Xa to the phospholipid surface. The K_d of factor Xa and the phospholipid mixture used in these experiments is about 10^{-6} M in the absence of factor Va and about 10^{-11} M in presence of an excess of factor Va [13,14]. The concentration of factor Va in our experiments therefore determines the amount of factor Xa bound to the phospholipids. That the pentasaccharide does not inhibit at high factor Va concentrations therefore means that is does not inhibit factor Xa bound to phospholipid and factor Va. Because the pentasaccharide does inhibit thrombin formation in plasma that is triggered by either tissue factor or by contact activation, it may be concluded that under these circumstances a rate limiting amount of factor Xa occurs in free form. Consequently, free factor Xa must be on the main pathway of thrombin generation in plasma

under our experimental conditions, and there is no reason to assume that there is a close interaction between the factor IXa - Factor VIIIa complex (intrinsic system) or the factor VII(a)-TF complex (extrinsic system) and the prothrombinase complex on the same surface when either human brain thromboplastin or phospholipid vesicles are used as a source of phospholipid.

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