Experimental hypercoagulable state induced by Factor X: Comparison of the nonactivated and activated forms

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Infusions of 630 U. of purified nonactivated Factor X failed to induce stasis thrombi in rabbits. In contrast, 5 U. of purified activated Factor X produced massive thrombi in the jugular veins of rabbits. Admixture of activated Factor X with 2 μg of phospholipid before infusion reduced tenfold the amount of activated Factor X required to produce stasis thrombi. Phospholipid also prolonged the hypercoagulability induced by activated Factor X from 10 to 200 seconds, but did not produce thrombosis itself nor make nonactivated Factor X thrombogenic. These data demonstrate for the first time that systemic hypercoagulability is not related to the absolute quantity of a circulating clotting factor, but rather to whether it is circulating in an activated form.

In searching for trigger mechanisms that might initiate intravascular coagulation in man, attention has frequently been focused on established clotting factors as thrombogenic agents. A several-fold increase in a circulating clotting factor could be expected to set the stage for a greater and more rapid explosion of coagulation enzyme kinetics. However, no experiments in man or animals have provided evidence to support this hypothesis.

To investigate this question, the thrombogenicity of purified Factor X in both its nonactivated and activated forms was examined. Factor X was chosen for this study because of its unique position in the clotting scheme and because in vitro kinetic data suggest that it is the pivotal enzyme in the elaboration of prothrombin activator. The results of these experiments demonstrate that the induced systemic hypercoagulability is not related to the absolute quantity of a circulating clotting factor, but rather to whether it is circulating in an activated form.

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Methods

Factor X, in its nonactivated form, was prepared from bovine plasma by the technique of Duckert, Yin, and Straub[1] modified in that the isolated Factor X obtained initially on the original DEAE-cellulose column was rechromatographed on another DEAE-cellulose column followed by gel filtration on a Sephadex G-200 column to achieve further chemical purification. The resulting fraction of nonactivated Factor X has a specific activity of 25,000 U. per milligram of tyrosine* and contains no detectable amounts of Factors I, II, V, VII, IX, activated XI, activated X, or thrombin.

Activated Factor X (autoprothrombin C) was isolated from Parke-Davis bovine thrombin by a previously described technique.[2] The activated Factor X is completely devoid of thrombin and other coagulation activities, including plasmin, tissue thromboplastin, and phosphatides.[2] Whether other activities not measured by these tests are present is not known. The specific activity is 22,000 U. per milligram tyrosine.

Methods adopted for the determination of activated Factor X and lipid phosphorus have been previously cited.[2] The cephalin employed in some experiments as a source of phospholipid was prepared from human brain.[3]

Sonicated platelet fractions were prepared as follows: 300 ml. of normal human blood were collected in 0.1M disodium ethylenediaminetetraacetic acid (EDTA) in a ratio of nine parts blood to one part anticoagulant with the use of a donor set. Unless otherwise stated, all containers were siliconized and operations carried out at 4° C. The blood was centrifuged at 1,000 × g for 5 minutes to obtain platelet-rich plasma. This plasma was centrifuged at 5,000 × g for 20 minutes. After centrifugation the supernatant fluid was decanted and the container rinsed with deionized water. The platelet-rich button at the bottom of the centrifuge tube was gently broken up in precooled 0.14M NaCl with the aid of a Vortex mixer. The platelet suspension was centrifuged at 5,000 × g for 10 minutes. The process of washing and resuspending the isolated platelets was repeated three times. Finally, the platelet-rich button was resuspended in 10 ml. 0.14M NaCl and sonicated with a Branson Sonifier at position seven for one minute or until a clear fraction was obtained. The resulting stock suspension contained 40 μg lipid phosphorus per milliliter.

The bioassay for the thrombogenicity of the infused fraction was modified from the standard technique for the production of stasis thrombi,[4] in that the volume of the infusate, in 0.14M NaCl, was 1 ml., the marginal ear vein infusion was completed in 2 seconds, and the jugular vein segment was ligated (unless otherwise specified) within 7 seconds after the start of the infusion. Healthy male albino New Zealand rabbits, each weighing approximately 1,500 grams and free of diarrhea, were used in the bioassay. Fasting refers to withdrawal of food, but not water, for 16 hours preceding the experiment.

Results

Thrombogenicity of nonactivated and activated Factor X. In our initial experiments, nonactivated Factor X, prepared from bovine plasma as described under Methods, was activated by either Russell's viper venom or trypsin with the subsequent removal of the activating enzymes by column chromatography. These preparations of activated Factor X were as thrombogenic as the activated Factor X prepared from Parke-Davis thrombin, and gave the same results in the in vitro Factor X clotting assay. Since activated Factor X was easier to prepare in large quantities from Parke-Davis thrombin, this material was used as a source of activated Factor X in these studies.

Infusions of nonactivated Factor X into fasted rabbits in amounts up to

*One unit of nonactivated Factor X can be defined as the amount present in 1 ml. of fresh normal human plasma. A unit of activated Factor X can be defined as the activity that would evolve from 1 ml. of normal human plasma when the precursor is fully activated by Russell's viper venom.

630 U. failed to induce stasis thrombi. In contrast, as little as 5 U. of activated Factor X was fully thrombogenic.

Influence of phospholipid (cephalin) on the concentration of activated Factor X required for thrombus formation. The admixture of phospholipid (2 μg lipid P) to activated Factor X immediately prior to infusion into fasted rabbits enhanced the thrombogenicity of the activated Factor X tenfold. In the absence of added cephalin, 5 U. of activated Factor X was required to induce stasis thrombi, whereas the amount of this factor necessary to produce thrombi was reduced to 0.3 U. in the presence of added phospholipid. Infusions of cephalin up to a level of 4 μg lipid phosphorus were not thrombogenic. The addition of cephalin to nonactivated Factor X did not confer thrombogenicity on the latter.

Admixture of the sonicated platelet fraction at a concentration of 2 μg lipid phosphorus with activated Factor X immediately prior to infusion resulted in the same pattern as that observed when brain cephalin was used. Infusions of the sonicated human platelet fractions alone at concentrations up to 4 μg lipid phosphorus were not thrombogenic when infused alone or together with nonactivated Factor X.

Influence of phospholipid on the duration of hypercoagulability induced by activated Factor X. As shown in Table I, the hypercoagulability induced by activated Factor X was extremely transient. Five units of activated Factor X created a hypercoagulable state that persisted for less than 10 seconds. However, when cephalin was added to the infusate, the duration of hypercoagulability was prolonged to 200 seconds. When the cephalin concentration was unchanged and the amount of activated Factor X reduced from 5 to 2.5 U. (not shown), hypercoagulability was prolonged from 5 to 80 seconds. This suggests that at a constant concentration of added phospholipid, the duration of hypercoagulability induced by activated Factor X is dependent upon the concentration of the latter. The sonicated platelet fraction (2 μg lipid phosphorus) had the same effect as cephalin in prolonging the duration of hypercoagulability induced by activated Factor X.

Table I. Effect of cephalin on duration of hypercoagulability of activated Factor X

Infusate		*Ligation delay after infusion (seconds)*	*Jugular vein thrombosis*
Activated X (U.)	*Cephalin (μg lipid P)*		
5	None	5	+
		10	0
5	2	5	+
		40	+
		80	+
		120	+
		160	+
		200	+
		240	0

Discussion

The data indicate that the infusion into a rabbit of an amount of activated Factor X equivalent to the amount of this activity that can be derived from 10 ml. of bovine plasma* was thrombogenic; whereas an infusion of nonactivated Factor X equivalent to the injection of more than 1 L. of bovine plasma was inert. Thus, results obtained in this investigation demonstrate, for the first time under controlled conditions, that at least one form of experimentally induced thrombosis is not related to the total quantity of a specific circulating clotting factor, but rather to whether or not it is circulating in an activated form. These conclusions are consistent with previous data from this and other laboratories,[5-10] and with the view that hypercoagulability should be defined as a state in which activated products of the coagulation process, normally absent from the circulating blood, are present intravascularly.[11]

This study also presented an opportunity to correlate some of the in vitro kinetic data concerning Factor X with the behavior of this clotting activity in vivo. Activated Factor X, although it cannot by itself convert fibrinogen to fibrin, is one of the essential components of prothrombin activator (prothrombinase), the principle responsible for the direct conversion of prothrombin to thrombin formed via either the intrinsic or extrinsic coagulation pathways. It has also been demonstrated in vitro that activated Factor X is the key moiety responsible for prothrombinase activity.[12] Lipids, as well as activated Factor X, calcium, and Factor V are required for the elaboration of prothrombin activator. The complexing of soluble activated Factor X with phospholipid, achieved in the presence of calcium, is instantaneous. This protein-lipid complex is particulate with a molecular weight size in excess of 200,000.[13] It has also been shown that the "thromboplastic activity" of phospholipid is dependent on the appropriateness of the surface charge of the lipid micelles rather than on the specific type of lipid involved.[14]

The finding that the thrombogenicity of activated Factor X was increased by cephalin without the latter being itself thrombogenic or conferring thrombogenicity on nonactivated Factor X indicates that there is an instantaneous formation of prothrombin activator in vivo when the activated Factor X-cephalin fraction is infused. The other factors, Factor V and calcium, that are already present in circulating blood do not represent rate limiting reactions in the formation of prothrombin activator. The specificity of the role of lipid is further emphasized by other experiments from this laboratory indicating that cephalin did not enhance the thrombogenicity of infusions of purified thrombin.[15]

The fact that cephalin prolongs the hypercoagulable state induced by activated Factor X remains to be explained. Although it has been suggested that activated Factor X is cleared by the liver,[7, 16] we have found in preliminary experiments that serum possesses an activity capable of neutralizing purified activated Factor X comparable to the inactivation of thrombin by antithrombin III. These observations are consistent with the fact that normal serum does not contain activated Factor X. The prolonged hypercoagulability of the activated

*Based on the finding that the bovine plasma Factor X level is one half that of human plasma.

Factor X-cephalin mixture suggests that its neutralization, whether by circulating inhibitors or clearance mechanisms, is different from activated Factor X alone. Thus, at low concentrations of lipid, the soluble activated Factor X is promptly inactivated; a result not so readily achieved when the activated Factor X is bound to lipid in the presence of calcium.

If the coagulation sequence is to be related to the thrombotic process, it will become necessary to demonstrate that in vitro clotting kinetics are applicable to the elaboration of fibrin intravascularly. The observations presented in this investigation concerning the thrombogenicity of activated Factor X and its augmentation by lipids is entirely in accord with known in vitro kinetic data on the function of Factor X in the coagulation sequence.

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