Utilization of a Continuous Flow Reactor to Study the Lipoprotein-Associated Coagulation Inhibitor (LACI) That Inhibits Tissue Factor

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A microperfusion system containing a glass capillary, the inner surface of which is coated with a phospholipid bilayer containing tissue factor, was used to explore the requirement for factors VIIa and Xa in the complex formed with the lipoprotein-associated coagulation inhibitor (LACI). Various combinations of factors VIIa, Xa, and LACI were perfused together or sequentially at a wall shear rate of 300 sec⁻¹; a final perfusion of factors X and VIIa was performed to evaluate the residual tissue factor activity. Factor Xa concentration at the outlet of the tube was determined using a chromogenic substrate. In the presence of factors VIIa, Xa, and LACI, complete inhibition of tissue factor was observed on both phosphatidylcholine (neutral surfaces) and on phosphatidyserine/phosphatidylcholine (acidic) surfaces; omission of factors Xa or LACI resulted in no inhibition. The absence of factor VIIa in the initial perfusion steps resulted in no inhibition on neutral surfaces whereas about 90% inhibition was observed on acidic surfaces. Initial perfusion with factor Xa, but not LACI, followed by the remaining protein components, resulted in an inhibitory complex. Thus, it appears that a tissue factor-factor Xa:LACI complex can form in the absence of factor VIIa on acidic surfaces; moreover, our data imply a tissue factor binding site for factor Xa, but not for LACI.

INHIBITION OF tissue thromboplastin activity by serum has been known for many years. Recently, several investigators have described the basic characteristics of this serum inhibitor, called either the lipoprotein-associated coagulation inhibitor (LACI) or the extrinsic pathway inhibitor. Tissue factor (TF), a transmembrane enzyme activator present in the plasma membranes of many cell types, initiates blood coagulation by enhancing the catalytic activity of factor VIIa toward its substrates, factor X and IX. By using purified coagulation proteins, LACI has been shown to inhibit TF by an interesting and indirect mechanism: activated factor X (Xa), a product of the TF pathway, forms a complex with LACI and it is this complex that is the true TF inhibitor. The inhibition of TF has been shown to be irreversible, thus precluding a direct proteolytic effect of the inhibitor on TF.

We have developed, as previously reported, a tubular reactor for studying coagulation reactions under controlled flow conditions. The reactor is a capillary whose inner wall consists of a phospholipid bilayer into which TF is inserted. Perusions can be performed over most of the range of physiologically relevant shear conditions. The perfusion system was shown to be simple to use and very reproducible.

In this report, we use the unique advantages of an immobilized enzyme in a flowing system to investigate the mechanism by which LACI inhibits the direct activation of factor X by TF:VIIa and, in particular, the order in which the reactants form the inhibitory complexes. Our data suggest that TF contains a binding site for factor Xa that is not involved in catalysis. The bound factor Xa, in turn, provides a LACI-binding site and thus is involved in the association of the Xa-LACI inhibitory complex with TF.

MATERIALS AND METHODS

The reactor is a small-diameter capillary whose inner wall is coated with a phospholipid bilayer containing TF. The formation of a stable, physically adsorbed layer was achieved using a modification of the technique of Watts et al. As described earlier, borosilicate glass micro-pipets from Fisher Scientific Co (Pittsburgh, PA) (0.27 mm internal diameter, 128 mm in length, no. 21-164-2A) were thoroughly cleaned in chromic acid and boiling detergent. The capillaries were filled with a 2 mmol/L phospholipid vesicle suspension containing 20 nmol/L TF in HEPES buffer (0.01 mol/L HEPES, 0.14 mol/L NaCl, pH 7.5) and incubated for 20 minutes at room temperature. The phospholipid vesicles (100 to 150 nm in diameter) consisting of either 30% (wt/wt) bovine brain phosphatidyserine and 70% egg phosphatidylcholine (TF:PS/PC) or 100% egg phosphatidylcholine (TF:PC) (both lipids from Avanti Polar Lipids, Pelham, AL) were prepared as previously described, and contained on average about 1.6 molecules of TF per vesicle, with about one half facing outward and therefore available.

The tubes were rinsed with a HEPES/albumin buffer (1 mg/mL bovine serum albumin) at a wall shear rate of 600 sec⁻¹ for 5 minutes (67 µL/min). The tubes were kept filled with buffer until used, and precautions were taken to avoid the introduction of air into the system on changing syringes.

A syringe pump (model 351; Sage Instruments, Cambridge, MA) was used to perfuse the capillaries at a wall shear rate of 300 sec⁻¹ (33.9 µL/min) with either the reaction mixture or various components thought necessary for the formation of the inhibitory complex. The reaction mixture used with TF:PS/PC coated tubes consisted of 100 nmol/L factor X, 10 nmol/L factor VIIa, and 5 nmol/L Ca²⁺. When TF:PC coated tubes were used, 600 nmol/L factor X was used because of the lower catalytic activity of this surface. All perfusions and reactions were performed at 37°C in an incubator.

In multi-step experiments capillaries were pre-perfused with one or two of the three components (10 nmol/L factor Xa, 10 nmol/L factor VIIa, and 0.5 nmol/L LACI) presumed necessary for inhibition. The capillary was then perfused with the other component(s) of the complete system, for a total of six combinations. The biologic activity of the TF was then assessed during a 20-minute perfusion containing factors VIIa and X. After each perfusion the capillaries were rinsed for 5 minutes at a flow rate of 67 µL/min, which is equivalent to 50 tube volumes. The requirement for Ca²⁺ was evaluated by performing rinses with and without this ion;
calcium ions were always present when proteins were perfused. The requirement for TF was demonstrated by coating a capillary with PS/PC vesicles prepared similarly to those containing TF but without the latter. The capillary was perfused for 30 minutes with a mixture of 100 nmol/L factor X and 10 nmol/L factor VIIa (both of human origin) in 5 mmol/L CaCl2. No detectable factor Xa was formed (data not shown).

Bovine coagulation factors were purified and quantified as previously described. Factor VIIa was prepared by cleaving factor VII with factor XIIa. Human coagulation factors were purified as previously described. Factor Xa was prepared via activation with the coagulant fraction of Russell's viper venom, followed by chromatography of the activated mixture on DEAE-Sephadex (Pharmacia, Piscataway, NJ). Prothrombin fragment 1 was prepared according to Forman and Nemerson. LACI was isolated from the conditioned medium of the human hepatoma cell line, HepG2. The purified inhibitor is functionally and antigenically similar to LACI in human plasma. When bovine proteins were used, all were of bovine origin with the exception of LACI; similarly, in experiments using human proteins, all were of human origin, including LACI.

A chromogenic assay was used to determine the activated factor X concentration emerging from the capillary during perfusion of the reaction mixture. The effluent was collected into microcentrifuge tubes containing 50 mmol/L EDTA (3 vol EDTA/1 vol sample) to inhibit further enzymatic activity. Fractions were collected every 5 minutes for up to 20 minutes, by which time a steady state (no change in factor Xa concentration with respect to time) had been attained in control experiments. A steady state is not reached in the presence of LACI (see Results). Samples were kept on ice until the end of the experiment when factor Xa concentrations were determined using an amidolytic technique: each sample tube was incubated in a block heater at 37°C and Spectrozyme FXa was added (0.5 nmol/L final concentration; American Diagnostica Inc, New York, NY). After 5 minutes the reaction was stopped by the addition of acetic acid (5% final concentration). The factor Xa activity was determined by measuring the absorbance (Gilford Spectrophotometer 240; Gilford Instruments, Oberlin, OH) of the free chromophore (paranitroaniline) at 405 nm. Under these conditions the absorbance was linear with factor Xa concentration to 5 nmol/L (final concentration). All reactions were diluted to less than 5 nmol/L. The presence of LACI in the reaction mixture at the concentrations used (Fig 1) were found not to affect the calibration curve.

**RESULTS**

**Effect of LACI Concentration on Factor Xa Production**

For these experiments bovine TF and factors VIIa and X were used. These factors were held constant while the concentration of LACI was varied from 0 to 500 pmol/L. The results obtained using TF/PS/PC are shown in Fig 1. In the absence of LACI, the concentration of factor Xa increased with increasing perfusion times, reaching a steady-state value approximately 5 to 10 minutes after initiation of the experiment. In these experiments we sampled the effluent every 5 minutes; however, we have performed experiments in which the effluent was sampled every minute and the steady state was maintained for times as long as 2 hours (not shown).

At LACI concentrations as low as 25 pmol/L, inhibition of factor Xa production was observed (Fig 1). The extent of inhibition increased significantly as the concentration of LACI increased. At an LACI concentration of 500 pmol/L, after an initial burst of Xa production, the outlet concentration of factor Xa was reduced to almost zero. The shape of the partially inhibited curves is in accord with the concept that the true inhibitor of TF is an LACI-Xa complex. Thus, as factor Xa production increases with time, the inhibitor complex concentration also increases, and so, consequently, does the extent of inhibition of TF.

The effect of 500 pmol/L LACI on TF/PC surfaces was also investigated. Because this surface produced much less factor Xa than did TF/PS/PC surfaces, as has been previously noted for a vesicular system, we used 600 nmol/L factor X for these experiments. Substantial inhibition by LACI was noted, although it was slightly less than that observed with TF/PS/PC (compare Fig 1 with Fig 3B).

**Investigation of Components Necessary for LACI Inhibition**

**Factor Xa and Ca2+**. Two-step experiments to test the requirement for various coagulation proteins in the LACI complex were conducted with TF/PS/PC surfaces, as shown in Fig 2. The general procedure involved pre-perfusing the system with given component(s), rinsing the capillaries with 50 tube vol of buffer, and then measuring the residual TF activity by perfusing the system with factors VIIa and X. In accord with previously published data, when the first perfusion contained LACI, factor Xa, and factor VIIa; or LACI, factor X, and factor VIIa and the rinse contained CaCl2, the production of factor Xa in the second perfusion was inhibited. In contrast, when the first perfusion mixture contained either LACI alone, LACI and factor X, or LACI and factor VIIa, normal levels of factor Xa at the tube outlet were measured in the second perfusion (Fig 2). As shown in Fig 2, factor Xa could be added directly to the perfusate or could be generated in situ from factor X. Thus, the tube reactor behaved similarly to a closed system with regard to factor Xa.

The intermediate rinse with buffer containing 5 mmol/L CaCl2 did not reduce the effectiveness of the inhibitory complex; however, the absence of CaCl2 in the rinse buffer resulted in reactivation of TF as manifested by the appear-
Fig 2. Effect of pre-perfusions with components (100 nmol/L factor X, 10 nmol/L factor VIIa, 10 nmol/L factor Xa, 0.5 nmol/L LACI) as indicated on the biologic activity of the TF:PS/PC surfaces as assessed during perfusion of the reaction mixture (100 nmol/L factor X, 10 nmol/L factor VIIa, 5 mmol/L Ca²⁺). In between perfusions the reactor was rinsed with a 50 tube vol rinse containing Ca²⁺. The control curve represents a capillary that was pre-perfused with only buffer.

Fig 3. Effect of pre-perfusions with 10 nmol/L factor Xa, 0.5 nmol/L LACI or 10 nmol/L factor Xa, 0.5 nmol/L LACI, 10 nmol/L factor VIIa on the biologic activity of the TF containing surfaces consisting of PS/PC (A) and PC (B) as assessed during perfusion of the reaction mixture (100 nmol/L factor X, 10 nmol/L factor VIIa for TF:PS/PC and 600 nmol/L factor X, 10 nmol/L factor VIIa for TF:PC). In between perfusions the reactor was rinsed with a 50 tube vol rinse containing Ca²⁺. The control curve represents a capillary that was pre-perfused with only buffer.

Sequential Requirements for Components Involved in LACI Inhibition

To investigate further the formation of the inhibitory complex, we performed multi-step perfusions, interspersed with two Ca²⁺-containing rinses. The final perfusion always consisted of the full complement of factors necessary for activation of factor X to occur (factors X and VIIa in the presence of 5 mmol/L Ca²⁺).

When the first perfusion consisted of LACI with or without factor VIIa and the second contained factor Xa, no inhibition was noted in the final step (Fig 4A). But, when the first perfusion contained LACI and factor Xa, marked inhibition of the final stage was noted for TF:PS/PC surfaces. This experiment shows that LACI has little or no affinity for the TF-VIIa complex in the absence of factor Xa.

In subsequent experiments, the first perfusion consisted only of factor Xa and/or factor VIIa. After the usual formation of factor Xa. The results are shown in Fig 3 for TF:PS/PC and TF:PC surfaces. As shown, the PS/PC and PC surfaces behave differently. With respect to PS/PC, pre-perfusion with factors Xa, VIIa, and LACI led to inhibition of factor Xa production in the second stage (Fig 3a). When factor VIIa was omitted from the initial perfusion, we observed the initial rate of factor Xa formation to be similar to the control experiment. However, inhibition of the system increased with perfusion time, and reached about 80% at 20 minutes.

These results contrast directly with those obtained with TF:PC surfaces where normal levels of factor Xa production were observed following a pre-perfusion of factor Xa and LACI. However, when factor VIIa was included in the pre-perfusion step marked inhibition was noted (Fig 3B). This observation is consistent with the concept that the inhibitory complex binds less tightly to TF:PC than to TF:PS/PC. When factor VIIa was included in the pre-perfusion, a complex was formed that is, by implication, more stable than the (putative) TF-Xa-LAC1 species.
Ca²⁺-containing rinse, the system was then perfused with LACI and either factor Xa or VIIa; after a second similar rinse, the system was perfused with the complete reaction mixture for assessment of TF activity. A very significant inhibition of factor Xa production was noted (Fig 4A). This experiment indicates that factor Xa was bound to the TF-containing phospholipid surface in the first perfusion. To confirm that the binding site was, indeed, TF, similar experiments were performed in the presence of 5 μmol/L prothrombin fragment 1, which has previously been shown to displace factor X from PS/PC surfaces. This ligand was included along with factor Xa in the first stage of the experiment. There was no discernable effect noted on inclusion of this lipid-binding ligand (Fig 4A). This finding suggests that factor Xa is not nonspecifically bound to the lipid surface and then diffusing to TF during subsequent manipulations.

The experiments above suggest an independent binding site on TF for factor Xa. To investigate this phenomenon further, we performed similar studies using TF:PC because it has been shown that factors VIIa, Xa, and X do not bind to this membrane. When either factor VIIa or Xa was included in the first stage, the results were similar to those obtained using TF:PS/PC; namely that these ligands appear to bind independently to TF. The only difference between these surfaces was manifested when the first perfusion contained factor Xa and LACI, and the second contained factor VIIa. In accord with the experiment depicted in Fig 3B, part or all of the Xa-LACI complex did not bind tightly to the immobilized TF on TF:PC, in contrast with the finding observed using TF:PS/PC (Fig 3A). An experiment was performed to determine if the Xa-LACI complex was lost from the surface or if only factor Xa remained bound to TF. A TF:PC coated capillary was initially perfused with factor Xa and LACI, and then (after a rinse step) perfused with factor VIIa and LACI. The subsequent biologic activity of TF as assessed by perfusion with factor VIIa and X was nil, suggesting that the inhibitory complex was formed (data not shown). This could only have been possible if factor Xa remained behind after the first perfusion.

**Inhibition Using All Human Proteins**

To confirm that these results are applicable in an all-human system, key experiments were performed using human TF:PS/PC and factors VIIa and X. In accord with the known decrease in the affinity of human Xa for LACI, and the fact that the wall concentration of TF was three times higher, incomplete inhibition of factor Xa production was observed at 500 pmol/L LACI (Fig 5). However, when the LACI was increased to 5 nmol/L, complete inhibition of factor Xa production was noted.

Pre-perfusion of factors Xa, VIIa, and LACI led to complete and stable inhibition as shown in Fig 6. Further, when factor Xa was pre-perfused, followed by LACI and VIIa, complete inhibition was also noted, thus indicating that human TF also has a factor Xa binding site. In addition, and
following concentrations: 5.0 nmol/L LACI, 10 nmol/L factor Xa. The first and second perfusions are as indicated and were at the factor Vlla, 5 mmol/L Ca2+. All human proteins. Components in rinsed with a 50 tube vol rinse containing CaZ+.

We have used a continuous flow capillary reactor to study the mechanism by which LACI inhibits the activation of factor X by TF:VIIa. These experiments were performed at a wall shear rate of 300 sec\(^{-1}\), typical of medium-sized arteries and veins.\(^{19}\) We chose this wall shear rate because preliminary experiments did not show marked differences in the range of 60 to 1,200 sec\(^{-1}\), and 300 sec\(^{-1}\) proved to be an experimentally convenient wall shear rate.

The inner wall of the capillary contained a phospholipid bilayer composed of either a 30/70 weight ratio of phosphatidylserine/phosphatidylcholine or pure phosphatidylcholine into which TF was inserted. With both surfaces, we observed that the concentration of factor Xa produced at the outlet of the tube was progressively inhibited by the presence of LACI in the perfusates; concentrations greater than 500 pmol/L LACI virtually eliminated the ability of TF to activate factor Xa alone, rinsed with Ca\(^{2+}\)-containing buffer, and then perfused with factor VIIa and LACI. Complete inhibition was then noted when the system was perfused with factors X and VIIa. Because TF bound factor Xa with both PS/PC and PC surfaces, we believe the evidence favors a specific binding site on TF. Further evidence for this view comes from the experiment in which 5 pmol/L prothrombin fragment 1 was perfused along with the factor Xa (Fig 4A). The inclusion of this ligand, which has been shown to displace factor X (and presumably factor Xa) from PS/PC vesicles,\(^{10}\) did not affect subsequent results. This site must be of high affinity because washing with 50 tube vol of buffer did not decrease the functional activity of this species as measured by support of LACI-inhibition. Occupancy of this site cannot be obligatory in TF-dependent catalytic cycles because its tight binding would clearly result in inhibition of product formation. Furthermore, this site might be related to the recently described factor Xa binding sites on endothelial cells and monocytes.\(^{22,21}\)

An interesting difference was noted between PS/PC and PC surfaces. When TF was reconstituted with the former, the Xa-LACI complex persisted after a rinse with Ca\(^{2+}\)-containing buffers (Figs 3 and 4), whereas when PC was used the Xa-LACI complex did not interact in a stable manner, at least as evidenced by lack of subsequent inhibition. It is clear that Xa-LACI binds more tightly to TF:PS/PC than to TF:PC. This is reminiscent of the binding of factors VII and VIIa to TF, which is tighter to TF:PS/PC than to TF:PC.\(^{9}\) The reason for these differences is unknown. It is noteworthy that when factors VIIa and Xa were present together, the subsequent addition of LACI resulted in a complex that was stable to the washout perfusions (Fig 4). This was true irrespective of the lipid composition. This finding suggests that factor VIIa participates in the formation of a quaternary complex, which could lead to a more stable species by contributing additional energy to the overall binding. The reduced level of inhibition in the absence of factor VIIa in pre-perfusion steps suggests that the Xa-LACI complex is not completely inhibitory and that the full, inhibitory species involves factor VIIa. Because factor VIIa is required to evaluate the activity of TF, this suggestion is difficult to verify. Nonetheless, it does appear that a slow reorientation of the Xa/LACI complex occurs in the presence of factor VIIa (see Fig 3A and 6).

In summary, the continuous flow reactor has been used to explore the mechanisms by which human LACI inhibits TF in a bovine system as well as an all-human system. This technique allows independent validation of previous findings as well as insights into the proposed action of LACI not easily

**DISCUSSION**

The data shown in Figs 4 and 6 indicate that TF has an independent binding site for factor Xa. In essence, this was shown by experiments in which the reactor was perfused with factor Xa alone, rinsed with Ca\(^{2+}\)-containing buffer, and then perfused with factor VIIa and LACI. Complete inhibition was then noted when the system was perfused with factors X and VIIa. Because TF bound factor Xa with both PS/PC and PC surfaces, we believe the evidence favors a specific binding site on TF. Further evidence for this view comes from the experiment in which 5 pmol/L prothrombin fragment 1 was perfused along with the factor Xa (Fig 4A).
investigated using standard techniques. The proposed final quaternary complex involves the direct Ca\(^{2+}\)-dependent interaction of factor VIIa and factor Xa on two separate domains of TF; LACI appears to interact predominantly with the immobilized factor Xa and not TF. In contrast to previous suggestions, LACI and factor Xa need not associate in the fluid phase before their interaction with TF. Interestingly, in the absence of factor VIIa, LACI could form a stable complex with TF:Xa sites only on surfaces containing phosphatidylserine. The inhibitory complex was stable and there was no evidence of displacement of the complex from the immobilized TF. In agreement with previous findings, the final complex was reversible in the sense that Ca\(^{2+}\)-free rinses restored the full activity of the immobilized TF, again excluding the possibility of a direct proteolytic alteration of TF as the mechanism of inhibition.

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