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FACTOR IXa PROTECTS ACTIVATED FACTOR VIII AGAINST INACTIVATION BY ACTIVATED PROTEIN C

Rogier M. Bertina, Rosemiek Cupers and Aat van Wijngaarden

Haemostasis and Thrombosis Research Unit, Leiden University Hospital, 2333 AA Leiden, The Netherlands

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SUMMARY: The effect of activated protein C on human coagulation factor VIII was evaluated by studying its effect on the intrinsic factor X activation using a system of purified coagulation factors (factor IXa, factor X, factor VIII, activated protein C). Activated protein C had no effect on the activation of factor X by factor IXa in the absence of factor VIII. In the presence of thrombin activated factor VIII the rate of factor X activation was decreased by activated protein C in a dose dependent way. The presence of factor IXa during the preincubation of factor VIII with activated protein C was found to protect the factor VIII against inactivation. The results suggest that activated protein C and factor IXa compete for the same part of the factor VIII molecule. © 1984 Academic Press, Inc.

Protein C is the zymogen of a serine protease which participates in the blood coagulation system and plays an important role in the regulation of fibrin formation and degradation (1). Protein C is converted into an active protease (activated protein C, APC) by thrombin (2). The rate of activation is greatly enhanced by the addition of thrombomodulin, a protein present on the surface of endothelial cells (3,4). Activated protein C significantly prolongs the Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) of human plasma (5). This prolongation has been explained by the inhibitory action of APC -presumably by proteolytic degradation -on (activated) factor V and (activated) factor VIII (6,7).

Factor VIII is another plasma protein involved in blood coagulation. In plasma it circulates mainly as a complex with a high molecular weight protein known as the Von Willebrand Factor (VWF). So far attempts to isolate this trace-protein from human plasma have only been partially successful (8,9). Its function in blood caogulation is that factor VIII after activation greatly enhances the rate of conversion of factor X by factor IXa (10). The inhibitory

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action of APC on (activated) factor VIII has been demonstrated using factor VIII clotting assays for the estimation of residual factor VIII activity (7,11). More recently it has been shown that APC catalyses a proteolytic degradation of several protein bands in a partially purified factor VIII preparation (12).

In the present report we studied the effect of APC on the factor VIII activity in a system of purified coagulation factors (activated factor IX, factor X and factor VIII). It was found that APC is a rather inefficient inhibitor of activated factor VIII and that activated factor IX (factor IXa) protects activated factor VIII against inactivation by APC.

METHODS & MATERIALS

Isolated coagulation factors

Human factor X was prepared using a minor modification of a method previously described (13). Human factor IXa was prepared by complete activation of purified factor IX with factor XIa (14); factor IXa was further purified by gelfiltration on Sephadex-G200 and concentrated by DEAE-Sephadex A50 chromatography; the preparation was stored at -20°C in 50% glycerol. Factor VIII was prepared by gelchromatography of cryoprecipitate on Bio Gel A15m in a buffer containing 20 mM sodium citrate, 150 mM NaCl and 10 mM benzamidine (pH 7.0); this preparation which has a specific coagulant activity of 40-80 Units/mg, still contains the VWF, which however does not interfere with the stimulatory effect of factor VIII on the factor IXa catalysed factor X activation (Mertens K and Bertina RM, unpublished observations). Factor VIII activity/factor VIII antigen ratios vary between 1.0 and 2.0, indicating that minor activation of the factor VIII might have occurred. Factor VIII preparations were dialysed against 50 mM Tris-Cl (pH 7.5), 100 mM NaCl directly before use in the kinetic experiments. Human thrombin was prepared as previously described (15). Activated protein C was prepared by activation of human protein C (16) with thrombin. After completion of the reaction, which was followed by the increase in amidolytic activity towards S-2366, activated protein C was separated from the thrombin by chromatography on DEAE-Sephadex and stored at -20°C in 50% glycerol. Activated protein C preparations migrate as one single band after SDS-PAGE (non-reduced) and have potent anticoagulant activity (estimated from the prolongation of the APTT asssay). Molar concentrations of the isolated proteins were calculated either from the molar extinction coefficients at 280 nm (factor X, protein-C, activated protein C) or from active site titrations with NPGB (thrombin, factor IXa).

Phospholipid vesicles were obtained as previously described (17) or by sonication of equimolar mixtures of L-α-phosphatidyl-L-serine (PS) and L-α-phosphatidylcholine (PC) (18). Phospholipid concentrations were determined by phosphate analysis after $HClO_4$ combustion (19).

Activated factor VIII was prepared by adding thrombin (0.15nM) to 500 µl of factor VIII (1-2 Units/ml). After 2.5 min at 37°C the activation reaction was stopped by addition of the specific thrombin inhibitor hirudin (20 Units/ml). Suitable aliquots of the activated factor VIII were added within 30 seconds to the incubation mixture in which factor X activation had to be measured (see measurement of factor X activation).

Measurement of factor X activation

To obtain stable phospholipid aggregates phospholipid vesicles were incubated in siliconized glass tubes for 10 min at 37°C with 16 mM $CaCl_2$, in 0.6 times the final volume of a buffer containing 50 mM Tris-Cl, 100 mM NaCl (pH 7.5). To these aggregated phospholipids were added factor IXa and activated factor VIII before the reaction was started with the addition of factor X. The final volume was 0.4 ml. All isolated factors had been dialysed against 50 mM Tris-Cl (pH 7.5), 100 mM NaCl prior to use. At different time intervals 50-100 µl samples were withdrawn from the incubation mixture for factor Xa analysis (see below).

Spectrophotometric factor Xa assay

50-100 µl samples were added to a buffer containing 50 mM Tris-Cl (pH 8.0), 100 mM NaCl, ovalbumin (0.1 mg/ml) and 5 mM EDTA (final volume 0.9 ml). After the addition of 100 µl of a 2.2 mM solution of S-2337, the increase in A-405 nm was recorded in a spectrophotometer (at 37°C). From the ΔA-405/min the factor Xa concentration in the sample was calculated using a calibration curve made with an active site titrated preparation of factor Xa.

Ovalbumin (5x recristallized) was obtained from Koch-Light laboratories, Colnbrook, Bucks., U.K. The chromogenic susbtrates N-benzoyl-L-isoleucyl-L-glutamyl (piperidyl)-t-glycyl-L-arginine-p-nitroanilide hydrochloride (S-2337) and H-pyro-glutamyl-L-prolyl-L-arginine-p-nitroanilide hydrochloride (S-2366) were products of Kabi-Diagnostica, Stockholm, Sweden.

Tris (TRIZMA-base), hirudin, L-α-phosphatidyl-L-serine and L-α-phosphatidylcholine were purchased from Sigma Chemical Co., St. Louis, MO, USA.

RESULTS

Factor IXa catalyses the conversion of factor X into factor Xa in the presence of phospholipids and Ca^{2+}-ions. This reaction is extremely slow: ~ 0.01 mol Xa/mol IXa/min (10). APC at a rather high concentration does not interfere with this reaction, nor does it activate factor X itself (see Table-1).

When non-activated factor VIII is added to the system consisting of factor IXa, factor X, phospholipids and Ca^{2+} a sigmoidal shaped Xa generation curve is

TABLE 1: Effect of APC on the activation of factor X by factor IXa

Additions	V_i(mol Xa/mol IXa/min)
PS/PC, FX	0.0000
PS/PC, FX, APC	0.0000
PS/PC, FX, FIXa	0.0117
PS/PC, FX, FIXa, APC	0.0096

PS/PC vesicles (115 µM) were aggregated in the presence of $CaCl_2$ after which FIXa (3nM) and/or APC (33 nM) were added. The reaction was started by the addition of factor X (0.1 µM). Rates of factor Xa formation were calculated from the amount of Xa formed after 0, 30 and 60 min of incubation.

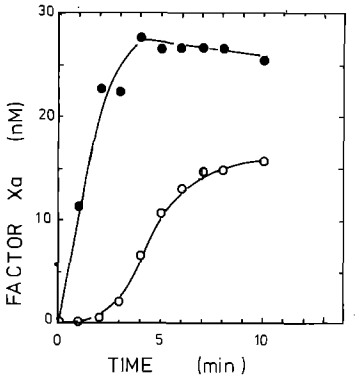

Figure 1
The effect of non-activated factor VIII and activated factor VIII on the activation of factor X by factor IXa.
Phospholipid vesicles (cephalin, 50 μM) were aggregated in the presence of $CaCl_2$ for 10 min, before the addition of factor IXa (0.67 nM) and non-activated factor VIII (o) or activated factor VIII (•). The reaction was started by the addition of 0.1 μM factor X; factor VIII: 0.9 U/ml; hirudin (20 U/ml).

obtained (see Fig. 1). Initially, the rate of factor X activation is identical to that observed in the absence of factor VIII (14); upon further incubation the rate of factor X activation increases by the activation of factor VIII by product Xa (14); at prolonged incubation the activated factor VIII is inactivated by factor Xa, which explains the appearance of a plateau in the Xa generation curve.

When in stead of non-activated factor VIII, activated factor VIII is added to a mixture of phospholipids, Ca^{2+}, factor IXa and factor X, the production of factor Xa is linear with time, which allows the calculation of an initial rate of factor Xa formation (see Fig.1). In the following experiments the concentration of the activated factor VIII was chosen so that it was rate limiting for the factor X activation.

Preincubation of activated factor VIII with APC in the presence of phospholipids, results in an inhibition of the inital rate of factor X activation (Fig. 2A). Less than 10 nM APC was necessary to obtain 50% inhibition. However, when activated factor VIII was incubated with APC in the presence of factor IXa, much higher APC concentrations needed to be added to obtain the same percentage of inhibition (Fig. 2A). In Fig. 2B the inhibitory effect of 20 nM APC on the rate of factor X activation was studied as function of the factor IXa

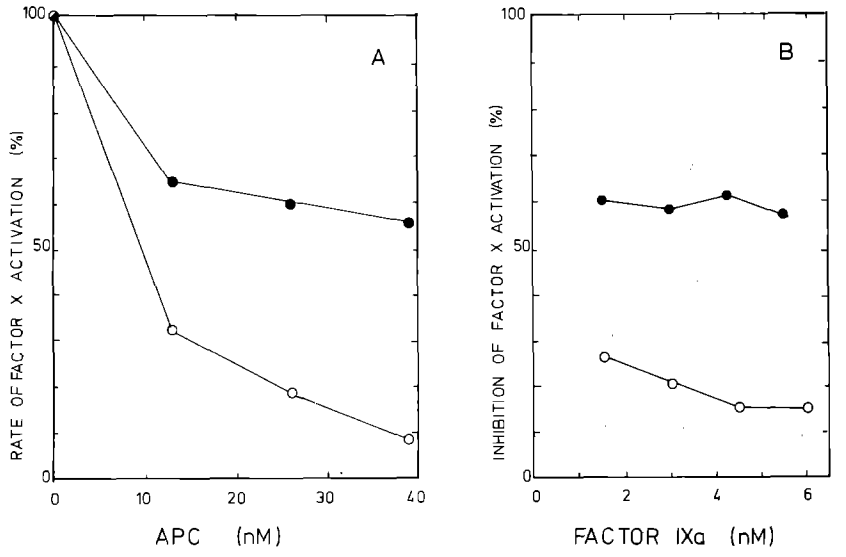

Figure 2A
The effect of APC on the activation of factor X by factor IXa in the presence of factor VIIIa.
PS/PC vesicles (100 μM) were aggregated in the presence of $CaCl_2$ for 10 min, and incubated with APC and activated factor VIII (+ hirudin) (0.18 U/ml) in the absence (o) or presence (●) of 3 nM factor IXa; after 2 min the reaction was started by the addition of factor X (0.1 μM)/factor IXa (3nM) or factor X (0.1 μM), respectively. Initial rates were calculated from the amount of factor Xa formed after 1, 2 and 3 minutes of incubation.

Figure 2B
The effect of the factor IXa concentration on the inhibition of intrinsic factor X activation by APC.
Conditions as in Fig. 2A; APC, 20 nM; factor VIII, 0.14 U/ml; factor IXa was either absent (●-●) or present (o-o) during the incubation of activated factor VIII with APC.

concentration. When factor IXa was present during the incubation of activated factor VIII with APC the percentage of inhibition was much lower than when factor IXa was not present. Moreover in the presence of factor IXa the actual percentage of inhibition tends to decrease at increasing factor IXa concentrations. These observations strongly suggest that the presence of factor IXa protects the activated factor VIII against inactivation by APC and that the protective effect of factor IXa is related to the factor IXa/APC ratio.

DISCUSSION

Preincubation of isolated factor VIII with APC in the presence of phospholipids and Ca^{2+}, results in a concentration dependent inhibition of the

activated factor VIII stimulated factor IXa activity (see Fig. 2A). It should be noted that in these experiments with isolated coagulation factors, the molar ratios of APC/factor VIII necessary to obtain significant inhibition, are rather high (about 10). One of the reasons might be that when compared to other activated vitamin K dependent factors, APC has been found to bind rather weakly to phospholipid membranes (20) and might need a cofactor to interact efficiently with these membranes. At present rather conclusive evidence has been presented that this indeed is the case (21) and that the presence of protein S, which is another vitamin K dependent plasma protein (22), is a necessary requirement for measuring the prolongation of PT and APTT by APC.

The presence of factor IXa clearly protects the activated factor VIII (Fig. 2A,B) against inhibition by APC. Moreover this protection seems to be more extensive at higher factor IXa/APC ratios. In the present models for the intrinsic factor X activator it is proposed that factor IXa binds to the activated factor VIII on the phospholipid surface and that this complex can catalyse factor X activation very efficiently (23). Apparently pretreatment of activated factor VIII with APC prevents the factor VIII to express this cofactor activity (binding of factor IXa). It is hypothesized now that factor IXa protects the activated factor VIII by binding to the same region of the factor VIII molecule were APC needs to bind. Such a hypothesis will explain that at constant APC concentration higher factor IXa concentrations will give a better protection. Similar results have been reported for the inactivation of activated factor V by APC (24). Activated factor V is the protein cofactor that greatly accelerates the activation of factor II by factor Xa. In this system factor Xa was found to protect activated factor V against inactivation by APC (6).

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