Isolation and partial purification of a novel anticoagulant from arteries of human umbilical cord

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An anticoagulant fraction was isolated from the homogenate of human umbilical cord arteries, using Sephadex gel filtration and DEAE-Sephacel chromatography. Analysis with dodecyl sulfate/polyacrylamide gel electrophoresis and inactivation studies using proteolytic enzymes indicate that the anticoagulant activity is associated with a polypeptide with an apparent Mr of 32000. The anticoagulant inhibits thromboplastin as well as factor Xa induced clotting but does not affect thrombin initiated fibrin formation. The anticoagulant inhibits the activation of prothrombin by the complete prothrombinase complex, by phospholipid bound factor X, but not by free factor Xa. The inhibition is instantaneous and independent of the incubation time over the whole range of concentrations tested. Therefore, the anticoagulant is unlikely to be a phospholipase or a protease. Its action does not resemble that of the plasma protease inhibitors, but it probably interferes with the phospholipid — clotting factor interactions.

The coagulation cascade, which has been studied extensively in the last decades, is now considered to be a multi-stage amplifying system of several linked proteolytic reactions in which an enzyme converts a zymogen into its active form (for review see [1]). The rate of these reactions is considerably enhanced by the presence of phospholipid and protein cofactors (factor V, factor VIII) [2, 3]. In vivo, the procoagulant reactions are controlled by several inhibitory mechanisms which prevent an explosive thrombotic event upon minor activation of the coagulation cascade.

Until now three major anticoagulant mechanisms have been recognized (for review see [4]). (a) The serine protease factor Xa and thrombin are inactivated as a result of their binding to antithrombin III [5] or, more efficiently, to antithrombin III - heparin complex. Both prothrombin activation and fibrin formation can thus be inhibited. Apart from antithrombin III several other plasma protease inhibitors exist (α2-macroglobulin, α1-proteinase inhibitor [6]). They all act in a time-dependent manner. (b) The discovery of protein C [7] has led to the understanding of another anticoagulatory mechanism. Protein C, once activated, functions as an anticoagulant through the selective proteolysis of the protein cofactors, factor Va [8] and factor VIIIa [9], thus inactivating prothrombinase and the factor-X-converting enzyme. (c) Plasmin cleaves fibrin 1 monomer, a product of the action of thrombin on fibrinogen, thereby preventing the formation of insoluble fibrin [10].

In this paper we report the isolation of a protein fraction from human umbilical cord arteries that instantaneously inhibits the activation of prothrombin by prothrombinase, i.e. factor Xa, factor Va, phospholipid and Ca2+. This inhibition leads to a prolongation of the prothrombin time. We demonstrate that the anticoagulatory mechanism is unlike those mentioned in this introduction.

MATERIALS AND METHODS

The chromogenic substrate D-Phe-Pip-Arg-NH-Np was purchased from AB Kabi Diagnostica, Stockholm, Sweden. DEAE-Sephacel, Sephadex G-100 and G-75 were products of Pharmacia. Chemicals for the analytical gel electrophoresis were from Bio-Rad. Human blood was collected by venepuncture in trisodium citrate (final concentration about 13 mM citrate) and centrifuged at 2000 g for 10 min at room temperature. The resulting plasma was recentrifuged at 10000 g for 15 min in order to obtain platelet-free plasma. A standard pool of platelet-free plasma was prepared by mixing plasma obtained from several healthy donors.

Homogenization of human umbilical cord arteries

Human umbilical cords were obtained within 15 min after delivery. The arteries were immediately perfused with ice-cold Tris/NaCl, subsequently prepared free from the Jelly of Warton and homogenized in Tris/NaCl using a whirl mixer, Braun MX32. A 10% homogenate (w/v) was fractionated as described in Results.

Gel electrophoresis

Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Laemmli [11] on slab gels containing 10% acrylamide, 0.27% N,N'-methylene-
bisacrylamide, and 0.1% sodium dodecyl sulfate. The gels were silver-stained according to the method of Merril et al. [12].

**Modified prothrombin time test (MPT test)**

The modified prothrombin time test (MPT test) was carried out as follows. In a siliconized glass cuvette 50 μl platelet-free plasma was stirred at 37 °C with 150 μl Tris/NaCl, 25 μl of a standard human brain thromboplastin dilution, and 25 μl Tris/NaCl (control) or 25 μl of a fraction of the arterial homogenate. After incubation for 3 min, coagulation was started at time zero with the addition of 250 μl Ca²⁺ buffer (80 mM NaCl, 20 mM CaCl₂ and 10 mM Tris/HCl pH 7.9). Fibrin formation was monitored optically with a Payton Dual Aggregation Module [13]. When factor Xₐ was utilized to initiate coagulation in the MPT test, thromboplastin was omitted and 25 μl purified factor Xₐ was added together with the 250 μl Ca²⁺ buffer to the diluted platelet-free plasma.

**Modified thrombin time test (MT test)**

This assay was carried out similarly to the Xₐ-initiated MPT test described above, with the exception that the Xₐ preparation was replaced by 25 μl of purified thrombin.

**Proteins**

Protease type I and trypsin were obtained from Sigma. Thromboplastin was prepared from human brain as described by van Dam-Mieras et al. [14]. Factor Xₐ, prothrombin and thrombin were purified from bovine blood as described by Rosing et al. [2]. Factor V was purified from bovine blood as described by Lindhout et al. [15]. Factor Vₐ was obtained by incubating factor V with thrombin [15]. Prothrombin concentrations were calculated using an Mₐ of 72000 and of 15.5 [16] and factor V concentration was calculated using an Mₐ of 330000 and of 9.6 [17]. Factor Xₐ and thrombin concentrations were determined by spectrophotometry Uvikon 810, and a reference curve, made with known amounts of purified thrombin, the amount of thrombin formed was calculated at different concentrations of the anticoagulant. The phospholipid was added as vesicles composed of Ole₂Gro-P-Ser and Ole₂Gro-P-Cho with a molar ratio of 20:80.

**RESULTS**

**Isolation**

Fractionation of the supernatant of a 10000 × g spin of an umbilical cord artery homogenate on Sephadex G-100 results in a reproducible specific elution profile (Fig. 1). The fractions affecting the coagulation system as measured with the modified prothrombin time test (MPT test) (see Materials and Methods) are indicated in Fig. 1. A procoagulant activity eluted with the void volume. This activity can only be detected in the presence of factor VII in the MPT test, as indicated by experiments in which human congenital factor-VII-deficient plasma was used (data not shown). Therefore this procoagulant must be considered to be tissue thromboplastin.

Certain fractions expressed a distinct anticoagulant activity. These fractions were pooled and further purified with DEAE-Sephalac chromatography (Fig. 2A). The anticoagulant appeared to bind to the DEAE-Sephalac at 50 mM NaCl and 50 mM Tris/HCl pH 7.9. Elution of the activity with a linear gradient of NaCl at pH 7.9 was achieved at 150–160 mM NaCl. The DEAE-sephacel fractions expressing anticoagulant activity were pooled and submitted to Sephacel G-75 gel filtration (Fig. 2B). The column (1.5 × 50 cm) was equilibrated with Tris/NaCl. The activity appeared in the fractions which correspond to molecular masses of 30–60 kDa. The MPT test is used as a quantitative assay for the determination of the amount of anticoagulant activity (Fig. 3). We define one unit as the anticoagulant activity that prolongs clotting time in the MPT test, using thromboplastin (final
Fig. 2. Chromatography of the anticoagulant on DEAE-Sephacel (A) and Sephadex G-75 (B). The pool containing the anticoagulant from the Sephadex G-100 column was applied to the DEAE-Sephacel. Elution was performed with a 200 ml linear gradient of 50–300 mM NaCl (---). Fractions (4 ml) were collected and assayed for $A_{280}$ (-----) and for anticoagulant activity in the MPT test, using thromboplastin (final concentration 95 µg protein/ml) as initiator of coagulation (●―●). The fractions with anticoagulant activity were pooled, concentrated and subsequently applied to Sephadex G-75 (B). Fractions (2 ml) were collected and assayed for $A_{280}$ (-----) and for anticoagulant activity (●―●). $V_o$ represents the void volume of the column.

Fig. 3. Dose response of the anticoagulant in the MPT test. Varying amounts of the anticoagulant were added to the MPT test. Coagulation was initiated with thromboplastin (final concentration 95 µg protein/ml). The control clotting time was 65 s.

Characterization

Several fractions of the Sephadex G-75 chromatography were tested in the MPT test and analysed with SDS-PAGE. The results (Fig. 4) suggested that the anticoagulant has a molecular mass of approximately 32 kDa. The association between the anticoagulant activity and the 32-kDa band was confirmed by slicing the polyacrylamide gel and subsequent elution of the proteins out of the slices with Tris/NaCl, containing 0.5 mg/ml bovine serum albumin. An anticoagulant activity is present only in the eluent of the slice that corresponds to the 32-kDa band. Furthermore, this activity is thermolabile at 56°C and demonstrates a similar dose–response relationship in the MPT test as the starting material.

The G-75 fractions, containing the peak anticoagulant activities were pooled and used for further characterization of the anticoagulant. Incubation of the anticoagulant at 56°C rapidly decreases its activity until after 2 min no activity can be measured. The anticoagulant loses its activity completely within 2 h upon incubation at 37°C with protease type I, whereas trypsin hardly inactivates the anticoagulant after an incubation period of 3 h (Fig. 5). The concentrations of protease type I and trypsin, used in these experiments, completely
Fig. 6. The effect of the vascular anticoagulant on the clotting times, induced in the MPT test by either thromboplastin, factor Xa or thrombin. The concentrations of the initiators of coagulation (thromboplastin 18 μg protein/ml, 1.5 nM factor Xa or 0.4 nM thrombin) were chosen such to give control clotting times of about 110 s (open bars). When factor Xa was used, phospholipid vesicles (final concentration 10 μM), composed of Ole2Gro-P-Ser/Ole2Gro-thrombin) were chosen such to give control clotting times of about 3.5 μg protein of the anticoagulant are represented by the shaded bars.

Fig. 7. The effect of the anticoagulant on prothrombin activation by Xa with Va, phospholipid and Ca2+. Xa with phospholipid and Ca2+ and Xa with Ca2+. The reaction mixtures contained: (A) 1 μM prothrombin, 1.0 nM Xa, 0.5 μM phospholipid and 10 mM CaCl2 with 12.0 μg/ml anticoagulant (●) and without anticoagulant (●); (B) 1 μM prothrombin, 10 nM Xa, 0.5 μM phospholipid and 10 mM CaCl2 with 2.4 μg/ml anticoagulant (●) and without anticoagulant (●); (C) 1 μM prothrombin, 75 nM Xa and 10 mM CaCl2 with 120 μg/ml anticoagulant (●) and without anticoagulant (●). At the times indicated samples were removed and thrombin was determined as described in Materials and Methods.

Results

The vascular anticoagulant was isolated by procedures described in Materials and Methods. The anticoagulant inactivated 2.5 nM thrombin in 15 min (data not shown). The amounts of protease type I and trypsin, carried over from the reaction mixtures to the MPT test, have no effect on the control clotting time.

Similar material could be obtained from human, bovine, rabbit and rat aorta and from a strongly vascularised tissue like lung. It could not be obtained from a poorly vascularised tissue like human diaphragm.

Mode of action

The MPT test is prolonged in the presence of the anticoagulant (Fig. 6) both when triggered with thromboplastin and when started with factor Xa. Thrombin-induced coagulation, however, is not inhibited.

Because of these findings we investigated the effect of the anticoagulant on the conversion of prothrombin to thrombin by factor Xa, factor Va, phospholipid and Ca2+. Under the experimental conditions mentioned, thrombin formation is inhibited by the anticoagulant in a dose-dependent way (Fig. 7A). The activation of prothrombin by factor Xa, phospholipid and Ca2+ in the absence of factor Va can also be inhibited by the anticoagulant (Fig. 7B). However, this inhibition is not observed if the activation takes place in the absence of phospholipid (Fig. 7C).

DISCUSSION

In this paper we describe the isolation procedure of a novel anticoagulant from a homogenate of human umbilical cord arteries. The anticoagulant has been discovered by its ability to prolong the clotting time in a prothrombin time test. The anticoagulant activity became measurable after Sephadex G-100 fractionation of the arterial homogenate. From further isolation procedures we assume this activity to be associated with a water-soluble substance(s), that carries an overall negative charge at pH 7.9.

Analysis of the Sephadex G-75 fractions (the final purification step we performed so far) with gel electrophoresis shows a positive correlation between the intensity of the 32-kDa band and the prolongation of the clotting time as measured with the MPT test. The identity of the 32-kDa band with the anticoagulant activity is demonstrated unambiguously since the anticoagulant activity can only be eluted from the site of the 32-kDa band on the polyacrylamide gel. From these findings and the fact that the anticoagulant rapidly loses its activity upon incubation at 56°C and proteolytic enzymes can destroy its activity, we assume the anticoagulant activity to be expressed by a single protein with an apparent molecular mass of 32 kDa.

Trypsin, in contrast to protease type I, is a poor inactivator of the anticoagulant. This suggests that the anticoagulant possesses a small number of lysine and arginine residues that are accessible for trypsin.

We studied the nature of the anticoagulant activity by initiating coagulation in different ways. Clotting, induced by either the vascular procoagulant, thromboplastin, or factor Xa, is inhibited by the anticoagulant; thrombin-induced clotting, on the other hand, is not. From these findings we conclude that the anticoagulant interferes with thrombin formation, not with thrombin action.

To study the anticoagulatory mechanism of the compound further we have used prothrombinase reconstituted from purified factors and thrombin [2]. Under the experimental conditions mentioned, the anticoagulant can inhibit the activation of prothrombin by complete prothrombinase (factor Xa, factor Va, phospholipid, Ca2+) and by phospholipid-bound factor Xa (factor Xa, phospholipid, Ca2+) but not by free factor Xa (factor Xa, Ca2+).

The time courses of prothrombin activation in the presence of the anticoagulant indicate an instantaneous inhibition of prothrombin activation, that remains constant in time. Therefore, we conclude that the anticoagulant does not act by phospholipase or by proteolytic activity. The fact that the activation of prothrombin by factor Xa and Ca2+ is not affected by the anticoagulant at all strongly indicates that the anticoagulatory mechanism of the vascular compound differs...
from that of the well known plasma protease inhibitors such as antithrombin III. Since Walker et al. [8] have demonstrated that activated protein C does not inhibit prothrombin activation by factor Xa, Ca2+ and phospholipid, it can also be concluded that our compound is not protein C either.

Preliminary binding studies indicate that the vascular anticoagulant probably interferes with the lipid binding of factor Xa and/or prothrombin. Whether the ability of the anticoagulant to inhibit prothrombin activation completely accounts for its prolonging effect on the prothrombin time remains to be established.

The fact that we could find this inhibitor in various types of arteries but not in a poorly vascularised tissue may indicate that we have found a physiological modulator of hemostasis and thrombosis, active at the vascular level.

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REFERENCES