964

TISSUE PLASMINOGEN ACTIVATOR INHIBITS THROMBIN-INDUCED AGGREGATION AND SHAPE CHANGE, BUT FACILITATES SECRETION, IN GEL-FILTERED PLATELETS ONLY. D. Blockmans, E. Van Houtte, J. Arnout, P. Mombaerts, D. Collen and J. Vermylen. Centre for Thrombosis and Vascular Research, University of Leuven, Belgium.

Prolonged administration of tissue plasminogen activator (t-PA) has caused bleeding problems in some patients, that did not necessarily correlate with a significant drop of fibrinogen levels. We have therefore evaluated the effect of t-PA on platelet function in vitro.

Incubation of gel-filtered platelets for one hour at 37°C with 180 µg/ml plasminogen and increasing concentrations of t-PA (50-1600 ng/ml) significantly inhibited shape change and aggregation induced by thrombin and the thromboxane mimetic U 46619 in a dose-dependent manner. In an EDTA milieu, which abolishes aggregation, a dual effect of t-PA and plasminogen was observed in the aggregometer: the thrombin- or U 46619-elicited initial decrease in light transmission, reflecting the disc-to-sphere transformation of platelets, was almost completely inhibited from 50 ng/ml t-PA upwards; the subsequent increase in light transmission, reflecting granule secretion, was however enhanced by small amounts of t-PA (up to 200 ng/ml). The latter finding was confirmed by direct measurement of secreted ATP: t-PA at concentrations up to 200 ng/ml enhanced thrombin- or U 46619-induced secretion. The amount of plasmin generated in the gel-filtered platelets-plasminogen-t-PA mixtures was quantified. The same amounts of plasmin, while also inhibiting the disc-to-sphere transformation of the platelets, did not enhance thrombin- or U 46619-induced ATP secretion. When whole blood or platelet-rich plasma or gel-filtered platelets resuspended in α_2-antiplasmin-depleted plasma was preincubated with t-PA, aggregation and/or shape change induced by ristocetin, arachidonic acid, the calcium ionophore A 23187, adenosine diphosphate, U 46619, thrombin, serotonin or platelet activating factor were not modified.

Our results suggest that in a purified system the effects of t-PA plus plasminogen on platelets are distinct from those of plasmin; it appears that low pharmacological concentrations of t-PA enhance the secretory responses to stimuli.

965

PLATELET FUNCTION IN PLASMA AT PHYSIOLOGICAL CALCIUM CONCENTRATION. THE USE OF VAC AS AN ANTICOAGULANT. R. van Gool, C.P.M. Reutelingsperger, G. Hornstra, and H.C. Hemker. Department of Biochemistry, University of Limburg, the Netherlands.

We have purified from human placenta an anticoagulatory protein (VAC, Mr= 32,000), which inhibits phospholipid dependent procoagulant reactions through a calcium dependent high affinity binding to procoagulant phospholipids. When tested in citrated platelet rich plasma (cPRP), VAC does not affect platelet aggregation and secretion in reponse to ADP, collagen and thrombin.
Purified VAC (0.05 µM, final concentration; f.c.) was used as an anticoagulant to prepare PRP (VAC-PRP). Platelet aggregation (optical density method) and release of newly absorbed ^{14}C-serotonin (5HT) in response to adenosine diphospate (ADP) were measured and compared with platelet responses in cPRP obtained simultaneously from the same donor.
The response of ADP stimulated platelets in VAC-PRP differs strikingly from that in cPRP. In the latter, platelets react with a dose-dependent primary aggregation, followed by a thrombin (IIa)-independent second wave of aggregation associated with 5HT-secretion. Platelets in VAC-PRP, however, demonstrate an increased primary aggregation in response to ADP, which is followed by a IIa-mediated second wave of aggregation and 5HT-secretion. Increasing the VAC concentration does not affect the primary aggregation response, but delayed the IIa-dependent secondary events in a dose-dependent way. At 0.5 µM VAC, platelets react to ADP (10 µM f.c.) with reversible aggregation only. No matter this high ADP-dose, secretion reaction does not occur. At this VAC concentration, epinephrine (5 µM f.c.) does not cause aggregation and 5HT-release at all, whereas in cPRP both reactions occur quite readily. Although in VAC-PRP, epinephrine retains its synergistic effect on ADP to aggregate platelets, no 5HT release was ever observed and the resulting aggregation was always reversible.
It is concluded that VAC is a suitable anticoagulant to investigate platelet function in the presence of physiological calcium concentration. Since platelet aggregation and release appear very different from results obtained in the usual way (cPRP, low calcium concentration) the physiological meaning of this latter method needs re-evaluation. Finally, our results cast severe doubt on epinephrine as an important platelet stimulant under physiological conditions.

966

CRYSTALLOGRAPHIC INVESTIGATION OF ANTITHROMBIN III.
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The plasma protein inhibitor antithrombin III in its native form has been crystallized using standard techniques. The crystals diffract to about 3Å and belong to space group $P4_12_12$ with cell parameters : a = b = 90.6Å, c = 380.7Å. The asymmetric unit contains three molecules of antithrombin III.

The self rotation function computed with the native data set indicates the presence of a non crystallographic three fold axis. Cross rotation function calculations using the model of the cleaved α_1-antitrypsin (H. Loebermann *et al.*, J. Mol. Biol. (1985) 177, 531) suggests tertiary structure similarities between the two plasma proteins. This is in agreement with the already described primary sequence homology of these glycoproteins but at variance with the model of active α_1-antitrypsin inferred from the previous studies on the cleaved molecule.

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967

INHIBITION OF FACTOR Xa AND THROMBIN BY ANTITHROMBIN III AND HEPARIN DURING HUMAN PROTHROMBIN ACTIVATION. P. Schoen, H.C. Hemker and T. Lindhout. Dept. of Biochemistry, University of Limburg, Maastricht, The Netherlands.

Prothrombin-catalyzed human prothrombin activation results in the generation of thrombin and meizothrombin- des F1 (MDF1) as was demonstrated by an immunoblot technique. The heparin-independent second order rate constants of inhibition of both thrombin and MDF1 were 3.7 x 10^{-5} $M^{-1}min^{-1}$, whereas the rate constant of inhibition of purified thrombin was 6.5 x 10^{-5} $M^{-1}min^{-1}$. In the presence of heparin the decay of amidolytic thrombin activity appeared to be bi-exponential and could be modelled by a 4-parameter equation. Fitting the experimental data to this equation gave the pseudo-first-order rate constants of inhibition, as well as the composition of the reaction with respect to the levels of thrombin and MDF1. The occurence of MDF1 rather than meizothrombin (MT) suggests that MT is very rapidly converted into prothrombin fragment 1 (F1) and MDF1, a "dead-end" product of prothrombin activation. At high prothrombin concentrations (1.0 µM) predominantly MDF1 and not thrombin is formed, indicating that MT itself and/or MDF1 cleaves at Arg156 - Ser157 to produce F1 and MDF1. Interestingly, heparin is unable to stimulate the inhibition of MT and MDF1 by AT III. We could demonstrate that MT and MDF1 have a low affinity, if any, for heparin.
The kinetics of the heparin-dependent inhibition of prothrombinase formed thrombin differ from those of purified thrombin added to the prothrombin activation reaction. A lower rate of inactivation of endogenous formed thrombin was observed and, moreover, could not be modelled as a simple random order bi-reactant enzyme-catalyzed reaction. These effects might be caused by the presence of prothrombin activation fragments.
Taking the factor Xa inactivation also into account, it appeared that the major effect of heparin on the thrombin generation was to enhance the inhibition of thrombin rather than that of factor Xa. When unfractionated heparin (UFH; mean Mr = 14000, 168 USP units/mg) was compared with a synthetic pentasaccharide (PS; Mr = 1714, 4000 anti-Xa U/mg) on basis of equal anti-Xa U/ml, UFH shortened the half-life time of factor Xa 190-fold, whereas PS shortened the half-life time 17-fold.