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FUNCTIONAL CHARACTERIZATION OF PROTEIN C INHIBITORS (PCI) FROM PLASMA AND URINE. M. Geiger (1), M.J. Heeb (1), B. Binder (2), and J.H. Griffin (1). Scripps Clinic and Research Foundation, La Jolla, CA U.S.A. (1), and Univ. of Vienna, Vienna, Austria (2).

Immunoblotting studies showed that human plasma and urine contain components that form heparin-dependent complexes with activated protein C (APC) and urokinase (UPA) and that the presence of either active enzyme decreases complex formation by the other (Geiger et al. 1986, Circ. 74:II-234). To investigate whether these findings are due to competition of APC and UPA for one or more heparin-dependent inhibitors, a functional assay for PCI was developed to study the time course of inhibition of protein C (PC) in purified or crude systems in the presence or absence of stimulating or interfering substances. Microtiter plates coated with monoclonal anti-protein C antibodies were incubated with diluted plasma supplemented with ^{125}I-PC. After activation of bound PC with Protac C, the wells were washed and then incubated with samples containing PCI in the presence or absence of heparin for varying times. The remaining amidolytic activity on the substrate S-2366 was determined as Δ A_{405}/time/cpm bound. Using plasma as a source of PCI, APC inhibition followed pseudo first order kinetics and a linear relation between the pseudo first order rate constant, k, and plasma concentration was observed from 6% to 40% plasma with k = 0.012/min at 20% plasma. A dose dependent increase in k was observed in the presence of heparin, with half maximal stimulation at 2 to 4 U/ml and maximal stimulation (k = 0.05/min at 20% plasma at heparin concentration $\geq$ 10 U/ml. Urinary PCI was partially purified using heparin Sepharose chromatography from which it coeluted at 0.35 M NaCl with UPA inhibitory activity. Partially purified urinary PCI inhibited APC and UPA, and competitions similar to those obtained with plasma PCI were observed. The effect of UPA on the inhibition of APC by PCI in plasminogen-depleted plasma and by urinary PCI was studied. In both systems, UPA caused a significant decrease in the inhibition of APC in the absence or presence of heparin. These results and our previous immunobloting data demonstrate competition of APC and UPA for common heparin-dependent PCIs in plasma and urine.

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IMMUNOLOGICAL SIMILARITIES BETWEEN PLASMA AND URINARY PROTEIN C INHIBITORS (PCIs) AND URINARY UROKINASE INHIBITOR (UKI). M.J. Heeb(1), F. Espana(1), M. Geiger(1), D. Collen(2), D.C. Stump(3), and J.H. Griffin(1). Scripps Clinic & Research Fndn, La Jolla, CA U.S.A.(1), Univ. Leuven, Leuven, Belgium (2) and Univ. of Vermont, Burlington, VT U.S.A. (3).

Plasma PCIs have similar MW (~ 57K), amino acid composition, and heparin dependence (Suzuki et al 1983, JBC 258:163) as urinary UKI (Stump et al 1986, JBC 261:12759). Urinary PCI of ~ 50K MW has a similar heparin dependence and urokinase (UK) competes with activated protein C (APC) for this PCI (Geiger et al 1986, Circ. 74:II-234). For comparison, three forms of PCI, one from urine and two from plasma, were purified, and each exhibited heparin-dependent UK and APC inhibitory activity and formed heparin-dependent complexes with APC. The APC-PCI complexes were visible on immunoblots (nondenaturing gels) developed using: A) monoclonal anti-UKI + ^{125}I-antimouse IgG; B) polyclonal anti-plasma PCI + ^{125}I-plasma PCI; and C) monoclonal anti-protein C + ^{125}I-protein C. The three forms of purified PCI were detected by methods A and B. Two new bands of APC-inhibitor complexes were seen upon incubation of plasma with APC in the presence of heparin, and the same pattern was visualized by methods A, B, and C. In the absence of heparin, only one APC-inhibitor band was visualized by methods A and B, but two bands were visualized by method C. Plasma immunodepleted of UKI by monoclonal anti-UKI-Sepharose showed no detectable antigen or complexes with APC as visualized by methods A and B. However, the UKI-depleted plasma contained components which formed a reduced amount of complexes with APC as visualized with protein C antibodies, i.e. method C. Heparin stimulates tenfold the PCI activity of normal plasma. Based on amidolytic assays of APC using S-2366, the UKI-depleted plasma was very deficient (< 15%) in heparin-dependent PCI activity, whereas the weak heparin-independent PCI activity was slightly reduced. This indicates that the majority of heparin-dependent PCI activity of plasma is immunologically related to UKI. These studies suggest that the two slightly different forms of plasma PCI, the urinary UKI, and the urinary PCI are very similar if not identical proteins and that plasma may contain a minor heparin-independent PCI which is not immunologically related to these proteins.

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A SECOND INHIBITOR OF ACTIVATED PROTEIN C (APC). F.J.M. van der Meer, N.H. van Tilburg, I.K. van der Linden, E. Briët, R.M. Bertina. Haemostasis and Thrombosis Research Unit, Leiden University Hospital, Leiden, The Netherlands.

The inactivation of APC in plasma, as measured with the chromogenic substrate S2366, follows, in the absence of heparin, pseudo-first order kinetics. The $t_{\frac{1}{2}}$ of about 20 minutes is independent of the APC concentration (31-500 nM) and increases linearly with the dilution of the plasma. These observations suggest that the concentration of the APC inhibitor in plasma is much higher than 500 nM, which is much higher than the concentration of 88 nM reported by Suzuki for the protein C inhibitor (PCI).

In the presence of heparin (5 IU/ml) the inactivation of APC becomes biphasic. Fast inactivation with an apparent $t_{\frac{1}{2}}$ of 8 minutes is followed by slower inactivation with a $t_{\frac{1}{2}}$ of 20 minutes.

Removal of PCI from the plasma with α-PCI antibodies (kindly provided by Dr. Suzuki) has no effect on APC inactivation in the absence of heparin. However, in this plasma APC inactivation could not be stimulated by addition of heparin (absence of fast phase of APC-inactivation). These data suggest the presence of two APC inhibitors in plasma: the heparin dependent PCI (PCI-I), earlier reported by Suzuki, and a sofar unknown heparin-independent inhibitor (PCI-II).

Further experiments showed that this PCI-II has a molecular weight of 60 kD and is different from the APC-binding protein reported by Kisiel. Using Heparin-Sepharose affinity chromatography we could separate PCI-II from both PCI-I and the APC binding protein. In this PCI-II preparation APC inactivation was accompanied by the formation of an APC-PCI-II complex of about 105 kD as demonstrated by immunoblotting with α-PC antibodies after SDS-PAGE. The identity of PCI-II is unknown; however, it is different from antithrombin III, heparin cofactor II, α_1-antitrypsin and α_2-antiplasmin.

The demonstration of the presence of two APC inhibitors in plasma will require a re-evaluation of the current functional assays for the APC inhibitor.

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IMMUNOLOGICAL ASSAYS FOR THE Ca(II)-DEPENDENT AND NONCa(II)-DEPENDENT CONFORMATIONS OF HUMAN PROTEIN Z (PZ). F.J.M. van der Meer, N.H. van Tilburg, S.R. Poort, E. Briët, R.M. Bertina. Haemostasis and Thrombosis Research Unit, Leiden University Hospital, Leiden, The Netherlands.

Human PZ was purified using bariumcitrate absorption and elution, DEAE-Sephadex chromatography and gelfiltration on Sephadex G-200. The purified protein was used for the development of a specific rabbit antiserum, which could be used in a electroimmunoassay (EIA). Using immunoaffinity procedures, two populations of antibodies were isolated from the antiserum: one against the Ca(II)-dependent conformation of PZ(Ca(II)PZAg) and one against the nonCa(II)-dependent conformation (nonCa(II)PZAg). These antibody populations were used for the development of specific and highly sensitive immunoradiometric assays for Ca(II)PZAg and nonCa(II)PZAg. In healthy volunteers PZ levels varied from 0.28 to 1.96 U/ml (mean value 0.99 ± 0.34 U/ml). The observed variation was much more extensive than for other vitamin K-dependent proteins. Repeatedly testing of individuals with low PZAg levels gave comparable results, excluding acute phase reactivity of PZ. The mean value for the Ca(II)PZAg/nonCa(II)PZAg ratio was 1.04 ± 0.27 (range 0.51 - 1.58).

Interestingly, PZ antigen levels were found to be severely reduced in 60 plasmas of patients stably treated with oral anticoagulants. At increasing intensity of treatment nonCa(II)PZAg decreased: 0.14 ± 0.04 (INR 2.6), 0.12 ± 0.04 (INR 3.0), 0.09 ± 0.03 (INR 3.4), 0.08 ± 0.03 (INR 3.8), 0.06 ± 0.01 (INR 4.2) and 0.06 ± 0.02 U/ml (INR >4.4).

The decrease in nonCa(II)PzAg was much more extensive than observed for the nonCa(II)Ag of other vitamin K dependent factors (factors II, VII, IX, X, protein C and protein S).

In general concentrations of Ca(II)PZAg were more reduced than those of nonCa(II)PZAg (ratio: ~ 0.7). This ratio was virtually independent of the intensity of oral anticoagulant treatment.