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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 ($\sim$ 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
$\sim$ 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 t½ 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 t½ of 8
minutes is followed by slower inactivation with a t½ 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 chromato-
graphy 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: $\sim$ 0.7). This ratio was
virtually independent of the intensity of oral anticoagulant
treatment.