SUPPLEMENTARY MATERIALS

Protein C Thr315Ala variant results in gain-of-function but manifests as type II deficiency in diagnostic assays

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Measurements of plasma protein C activity and antigen

The PC antigen (PC:Ag) level was tested with ZYMUTEST Protein C antigen assay kit (Hyphen BioMed, Neuville-Sur-Oise, France). The PC activity (PC:A) levels in plasma were determined by both amidolytic activity assay (Instrumentation Laboratory, Bedford, MA, USA) and aPTT assay using the Staclot Protein C Activity Assay kit (Diagnostica Stago, Asnieres, France) in accordance with the manufacturer’s instructions and as described.

Expression and purification of recombinant proteins

Both wild-type and the Thr-315 to Ala substitution (T315A) variant of PC were expressed in HEK-293 cells and purified to homogeneity as described previously. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10% polyacrylamide gel and stained with Coomassie Blue R-250 to verify homogeneity and quality of both PC derivatives under non-reducing and reducing conditions. The zymogens (0.5-1.0 mg) were converted to activated protein C (APC) by thrombin (25 μg) in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.4 (TBS) containing 5 mM EDTA for 2h at 37°C as described. Both wild-type APC and APC-T315A were separated from thrombin using an FPLC Mono Q column developed with...
a 40 mL linear gradient from 0.1 to 1.0 M NaCl. The properly γ-carboxylated APC derivatives were eluted at ~0.45-0.5 M NaCl as described. The concentrations of APC derivatives were determined from the absorbance at 280 nm assuming a molecular mass of 56 kDa and an extinction coefficient (E\text{\textsubscript{1cm}}\text{\textsuperscript{1%}}) of 14.5 and by stoichiometric titration of enzymes with known concentrations of recombinant protein C inhibitor (PCI) as described. The recombinant proteins including PCI, soluble TM (sTM), and W215A/E217A-thrombin (WE-thrombin) were expressed and purified as described by the cited methods.

Human plasma proteins factor Xa (FXa), prothrombin, antithrombin, factors V and Va were purchased from Hematologic Technologies Inc. (Essex Junction, VT). Human recombinant FVIII and FVIIIa were generous gifts from Dr. Philip Fay (University of Rochester, Rochester, NY). Human protein S was purchased from Enzyme Research Laboratories (South Bend, IN). α\text{\textsubscript{1}}-antitrypsin was obtained from Athens Research and Technology, Inc. (Athens, GA). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described. Recombinant tissue factor lacking the cytoplasmic domain (dcTF) was expressed in bacteria and incorporated into PC/PS vesicles as described. APTT reagent (Alexin) was purchased from Trinity Biotech (St. Louis, MO). Normal pooled plasma was purchased from George King Bio-Medical, Inc. (Overland Park, KS). Chromogenic substrates, Spectrozyme PCa (SpPCa) was purchased from American Diagnostica (Greenwich, CT) and S2238 was purchased from Kabi Pharmacia/Chromogenix (Franklin, OH) and the active-site probe, p-aminobenzamidine (PAB), was purchased from Sigma.

**Protein C activation**
The time course and concentration dependence of PC activation by the thrombin-TM complex was analyzed as described.\textsuperscript{1,2} Briefly, PC derivatives (1 $\mu$M) was incubated with thrombin (1 nM) in complex with sTM (10 nM) in TBS containing 2.5mM Ca\textsuperscript{2+}, 1.0 mg/mL BSA and 0.1% polyethylene glycol (PEG) 8000 (TBS/Ca\textsuperscript{2+}) at room temperature. At different time intervals, thrombin activity was quenched by 0.5 $\mu$M AT and the rate of APC generation was measured from the cleavage rate of SpPCa at 405 nm by a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) as described.\textsuperscript{1,2} The same procedures were employed to measure the concentration dependence of protein C activation by the thrombin-TM complex. The concentrations of APC derivatives in the reaction mixtures were determined by references to standard curves which were prepared by the total activation of PC derivatives with excess thrombin at the time of experiments.

**Cleavage of chromogenic substrates and Inhibition by PAB**

The steady-state kinetics of hydrolysis of SpPCa (7.8–1000 $\mu$M) by APC derivatives (4 nM) was measured in TBS/Ca\textsuperscript{2+} at room temperature as described.\textsuperscript{1} The affinity of wild-type and APC-315A for interaction with the active-site directed serine protease probe, PAB, was determined by incubating each protease (5 nM) with increasing concentrations of PAB (0-320 $\mu$M) in the presence of different fixed concentrations of S2266 (31–1000 $\mu$M) in TBS/Ca\textsuperscript{2+} at room temperature. The enzyme activities were measured from the cleavage rate of the chromogenic substrate as described above, and the $K_i$ values were determined by global fitting of data to a competitive binding equation as described.\textsuperscript{5}

**Inhibition by plasma inhibitors**
The reactivity of APC derivatives with plasma inhibitors was assessed by incubating the proteases at a final concentration of 20 nM APC with normal plasma at 37 °C. At indicated time points, aliquots were removed, and the residual amidolytic activity was determined using the chromogenic substrate SpPCa as described above. The half-life of each protease was calculated based on the time required to observe 50% decline in the amidolytic activity. The reactivity of APC derivatives with antithrombin, PCI, and α₁-antithrypsin was also analyzed in the purified system using an amidolytic activity assay as described.¹

**Statistical analysis**

Results are expressed as mean ± SD, and t-Tests (paired or independent) were used to assess data. Differences were considered statistically significant at p values of <0.05. All experiments were repeated at least three times.

**References**
