Ile73Asn mutation in protein C introduces a new N-linked glycosylation site on the first EGF-domain of protein C and causes thrombosis

Yeling Lu,[1,2] Padmaja Mehta-D'souza,[2] Indranil Biswas,[2] Bruno O. Villoutreix,[3] Xuefeng Wang,[1] Qiulan Ding,[1] and Alireza R. Rezaie[2,4]

[1]Department of Laboratory Medicine, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China; [2]Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA; [3]Université Lille, Inserm, Institut Pasteur de Lille, U1177 - Drugs and Molecules for Living Systems, F-59000 Lille, France and [4]Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

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Correspondence: *ALIREZA R. REZAIE*- ray-rezaie@omrf.org

QIULAN DING - qiulan_ding@hotmail.com

SUPPLEMENTARY MATERIALS

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[1]Department of Laboratory Medicine, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China; [2]Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City; [3]Univ. Lille, Inserm, Institut Pasteur de Lille, U1177 - Drugs and Molecules for Living Systems, F-59000 Lille, France; and [4]Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

Methods

Construction, expression, and purification of recombinant protein C derivatives

The expression of recombinant human wild-type protein C in human embryonic kidney (HEK-293) cells has been described previously.[1] The protein C derivatives including Ile73 to Asn (I73N) and Arg352 to Gln (R352Q) mutations in the protein C numbering system (R187Q, in chymotrypsin numbering)[2] were constructed and expressed using the same vector system as described.[1] All recombinant proteins were isolated from cell culture supernatants by a combination of immunoaffinity and ion exchange chromatography using the HPC4 monoclonal antibody immobilized on Affi-gel 10 and a Mono Q FPLC column, respectively, as described.[1] The zymogens (~1 mg) were converted to activated protein C (APC) by thrombin (50 μg) in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.4 (TBS) containing 5 mM EDTA for 2 h at 37°C and APC derivatives were separated from thrombin using a Mono Q FPLC column developed with a 25 mL linear gradient from 0.1 to 1.0 M NaCl. The properly γ-carboxylated APC derivatives were eluted at ~0.45 M NaCl as described.[1] The concentrations of APC derivatives were determined from the

absorbance at 280 nm assuming a molecular mass of 56 kDa and an extinction coefficient ($E^{1\%}_{1cm}$) of 14.5, and by stoichiometric titration of enzymes with known concentrations of recombinant protein C inhibitor (PCI) as described.[1] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% polyacrylamide gel and stained with Coomassie Blue R-250 to verify homogeneity and quality of both wild-type and mutant APC derivatives under both non-reducing and reducing conditions. Other recombinant proteins including PCI and soluble TM (sTM) were expressed and purified as described.[1,3]

Human plasma proteins including factor Xa (FXa), prothrombin, antithrombin (AT), protein S and factor Va (FVa) were purchased from Hematologic Technologies Inc. (Essex Junction, VT). Human protein S-deficient plasma and protein C-deficient plasma were purchased from Affinity Biological Inc. (Ontario, Canada). Normal pooled plasma was purchased from George King Bio-Medical, Inc. (Overland Park, KS). FVa Leiden, purified from the plasma of a homozygous patient was a generous gift from Dr. Charles Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described (21). APTT reagent (KONTACT) was purchased from Thermo Scientific (Middletown, VA). Chromogenic substrates, Spectrozymes PCa (SpPCa) was purchased from Biomedica Diagnostics (Stamford, CT) and S2238 was purchased from Kabi Pharmacia/Chromogenix (Franklin, OH). N-Glycosidase F (PNGase F) was purchased from New England BioLabs (Ipswich, MA).

Protein C activation

The initial rate of activation of protein C derivatives by thrombin was monitored in both the absence and presence of sTM as described.[4] In the absence of sTM, the time course of protein C (1 μM) activation by thrombin (10 nM) was studied at room temperature in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.4 (TBS) containing 1 mg/ml BSA, 0.1% PEG 8000 and 1 mM EDTA in 96-well assay plates. At different time intervals, thrombin activity was quenched by 0.5 μM of the AT-

heparin complex, and the rate of APC generation was monitored from the cleavage rate of SpPCa at 405 nm with a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). In the presence of sTM, the activation of protein C by thrombin (1.0 nM) was monitored in the presence of sTM (50 nM) in TBS containing 1 mg/ml BSA, 0.1% PEG 8000 and 2.5 mM Ca^{2+} (TBS/Ca^{2+}). The concentration of APC in reaction mixtures was determined by reference to a standard curve which was prepared by the total activation of protein C derivatives with excess thrombin at the time of experiments.

Cleavage of chromogenic substrates

The steady-state kinetics of hydrolysis of SpPCa (16–1000 μM) by APC derivatives (5 nM) was measured in TBS/Ca^{2+} at 405 nm at room temperature in a Vmax Kinetic Microplate Reader as described above. The K_m and k_{cat} values for the substrate hydrolysis were calculated from the Michaelis-Menten equation.

Analysis of thrombin generation in plasma

Thrombin generation (TG) assay was performed with Thrombinoscope (Fluoroskan Ascent (Thermo Fisher Scientific, Waltham, MA) using citrated human normal or protein C-deficient plasma reconstituted with either recombinant wild-type protein C or mutant protein derivatives (60 nM). The direct effect of APC on inhibition of thrombin generation was also evaluated. All reagents were obtained from Thrombinoscope, Stago. All samples were assayed in duplicate. Briefly, 20 μL of the PPP-Reagent Low [containing phospholipids and a low concentration of tissue factor (1 pM)] was added to 80 μL plasma followed by automatic delivery of 20 μL FluCa containing calcium and a fluorogenic substrate. Thrombin generation was monitored for 60 min and the resulting calibrated automated thrombogram was analyzed for thrombin generation parameters including lag time (min), peak height (Peak, nM), time to peak (ttPeak, min) and endogenous thrombin

potential (ETP, nM*min). In some experiments, TG assay was performed either in the presence of 2-5 nM sTM or in the presence of 5-10 nM purified APC derivatives.

Genetic analysis

Genomic DNA was extracted from peripheral whole blood using the QIAamp DNA blood purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Detection of genetic defects of the *PROC* was carried out by directly sequencing on an ABI 3700 sequencer (Applied Biosystems, Foster City, CA).

Anticoagulant assays

The anticoagulant activities of APC derivatives in the absence and presence of protein S were monitored both in purified and plasma-based assay systems as described.[4,5] In the purified system, the degradation of FVa by APC derivatives was evaluated as described. Briefly, FVa (2.5 nM) was incubated with increasing concentrations of APC derivatives (0-10 nM) on 25 μM PC/PS vesicles in TBS/Ca^{2+}. Following 10 min incubation at room temperature, the remaining FVa activity was determined in a prothrombinase assay as described.[4,5] Thrombin generation was monitored by an amidolytic activity assay using S2238 (100 μM). The same assay was used to monitor the inactivation of FVa by increasing concentrations of APC in the presence of protein S (50 nM) with the exception that incubation time was decreased to 1 min.

This assay was also used to evaluate the protein S concentration dependence of FVa degradation in the presence of a fixed concentration of PC/PS vesicles (25 μM) and the PC/PS concentration dependence of FVa degradation by APC derivatives in the presence of a fixed concentration of protein S (20 nM). The apparent dissociation constant ($K_{d(app)}$) for interaction of APC derivatives with protein S and PC/PS vesicles were determined from non-linear regression analysis of the saturable-dependence of the inhibitory effect of APC derivatives toward the cofactor activity of FVa in the prothrombinase assay as described.[5] The % inhibition of FVa

cofactor activity in the prothrombinase was plotted as a function of increasing concentration protein S and/or PC/PS vesicles.

The anticoagulant activities of APC derivatives were also evaluated in normal and protein S-deficient plasma by an aPTT assay using STart 4 fibrinometer (Diagnostica/Stago, Asnieres, France). In both cases, 0.05 mL TBS containing 0-10 nM APC was incubated with a mixture of 0.05 mL of plasma plus 0.05 mL aPTT reagent (CONTACT) for 5 min before initiating clotting by the addition of 0.05 mL $CaCl_2$ (25 mM) at 37 °C as described.[4,5]

Interaction with Na^+

The apparent dissociation constant ($K_{d(app)}$) for the interaction of Na^+ with either APC-WT or APC-R352Q (5 nM each) was determined from the effect of varying concentrations of Na^+ (NaCl) on the activity of each protease toward SpCa (100 μM) at room temperature in 0.02 M Tris-HCl, pH 7.4, 1 mg/mL BSA, 0.1% PEG 8000 lacking or containing 2.5 mM Ca^{2+} as described.[6,7] The $K_{d(app)}$ values were calculated from the hyperbolic increase in the rate of substrate hydrolysis as a function of increasing concentrations of NaCl. The ionic strength of the reaction buffer was compensated with Tris-HCl (pH 7.4) as described.[6,7]

N-Glycosidase F (PNGase F) treatment

The PNGase F treatments of wild-type and I73N mutant of protein C were performed according to a protocol provided by the manufacturer. Briefly, the protein C zymogen or APC derivatives (5 μg each) were combined with 1 μL Glycoprotein Denaturing Buffer (10X) in a total reaction volume of 10 μL followed by heating the reactions at 100°C for 10 min in order to denature the protein samples. Then, 2 μL of GlycoBuffer 2 (10X), 2 μL of 10% NP-40, and 2 μL of PNGase were added to each reaction and adjusted to a 20 μL total reaction volume. The reactions were incubated at 37°C for 1 h and analyzed by SDS-PAGE on a 12% gel.

Molecular modeling

The molecular models of the Gla-EGF1 domains of APC and protein S (built based on the x-ray structures of prothrombin, Gla-domainless APC and FVIIa) have been reported previously.[8,9] The pyDock computational program[10] was used to dock the two membrane binding regions of APC and protein S as described.[5] The Ile73 to Asn substitution was introduced in APC with the PyMol molecular graphic program (Schrodinger, Cambridge, USA). The GLYCAM server was used to build, graft and energy minimize a hybrid N-glycan at APC position 73 as described.[11] The computational manipulation was straightforward since this residue is fully solvent exposed in the x-ray crystal structure of APC. The figure was generated using PyMol.

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