

Supplemental Data

Blocking human protein C anticoagulant activity improves clotting defects of hemophilia mice expressing human protein C

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Running title: Blocking human protein C activity improves coagulation defects

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Materials and Methods

Human protein C and bovine thrombin were prepared as described previously.¹ Recombinant APC (Xigris) was from Eli Lilly. Spectrozyme PCa was from American Diagnostica. 1-Palmitoyl-2-oleoyl-phosphatidylcholine (PC), 1-palmitoyl-2-oleoyl-phosphatidylserine (PS), and 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (PE) were from Avanti Polar Lipids, Inc. Human endothelium derived EA.hy926 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum, L-glutamine and HAT (hypoxanthine, aminopterin, thymidine). Fluorescein labeled APC (FL-APC) was prepared with the Fluorescein-EX Protein Labeling Kit from Molecular Probes according to the manufacturer's instructions.

Quantitative reverse transcription PCR (qRT-PCR)

To detect the transcripts of *mProc* or *hPROC*, total RNA from mice liver was isolated using RNAiso Plus (Takara, Japan; NO.9108). The RNA was converted to cDNA using PrimeScript RT Master Mix (Takara; RR036A) to amplify various partial fragments of *PROC*, *Proc*, or mouse *F8* or *F9* mRNA. Primer sequences are as follows: human protein C forward primer GCCACCTGGGGAATTTCCG, reverse primer CCAAGCACTGGTCACCGTC; mouse protein C forward primer CATGTCCACCTGGGGAATATCT, reverse primer CTGTTGGCACGTCTGACCC; FVIII forward primer TGACCCTCCATGTCTCACTTAC, reverse primer GTGCATTTTAGGCCAGTCTCT; FIX forward primer

ATGCTGGTGCCAAGTTGGATT, reverse primer

CTCAGTGCAGGAACAAATTACCT; GAPDH forward primer

AGGTCGGTGTGAACGGATTTG, reverse primer GGGGTCGTTGATGGCAACA. qPCR

was performed using SYBR Green PCR Master Mix (Roche Life Science, #11051421) on the

Roche LightCycler480 Real-Time PCR System. Results were normalized to GAPDH RNA,

and the fold change was determined using the $2^{-\Delta\Delta CT}$ method. A statistical analysis was

conducted using GraphPad Prism 8 software.

Human protein C concentration measurement by enzyme-linked immunosorbent assay (ELISA)

Human protein C concentrations in the plasma of various mouse models and human were

measured using a matched-pair antibody set for ELISA of human Protein C antigen kit from

Enzyme Research Laboratories (South Bend, Indiana, USA), according to the manufacturer's

instruction.

Coagulation assays of mice plasma

For activated partial thromboplastin time (aPTT), mice plasma (50 μ l) was mixed with aPTT reagent (50 μ l; Sysmex, China; R9051), and the mixture was incubated for 5 min at 37°C.

Then, 25 mM CaCl₂ (50 μ l) was added to trigger the clotting reaction. Clotting time was

measured by a four-channel semi-automated coagulation analyzer SStart® (Stago). For

prothrombin time (PT), mice plasma (50 μ l) was added to the reaction cup and incubated for

5 min at 37°C, and then Neoplastine PT reagent (100 µl; Sysmex, China; R9055) was added to initiate the clotting reaction. Clotting time was measured by SStart®. For fibrinogen (FIB) measurement, the FIB reagent (Sysmex, China; R8051) was separately added to mice plasma (50 µl) and fibrinogen standard (50 µl) to initiate the reaction. The reaction was automatically carried out under 37°C for 5 min, and the clotting time was automatically recorded by SStart®. For thrombin generation assay, mice were anesthetized with 1% pentobarbital sodium solution, and maximum amount of blood was collected from the inferior vena cava. Platelet-rich plasma (PRP) was prepared and preserved under -70 °C. Mouse PRP (80 µl) was added to PP- Reagent LOW or thrombin standard (20 µl; Stago) for calibration. Each reaction was initiated with FluCa (20 µl; Stago) and incubated at 37 °C for 10 min. Fluorescence was read by using 360 nm excitation and 460 nm emission wavelengths with calibrated automated thrombogram (CAT) equipment (Stago). All the experiments were carried out in triplicate.

Platelet count of mice blood

Blood was collected from orbital venous plexus. 50 µl of the blood was analyzed for platelet number by an BC-5000Vet hematology analyzer (Mindray; Shenzhen, China).

Generation of mouse anti-human protein C and APC monoclonal antibodies

Mouse monoclonal antibody (mAb) against human protein C or APC was developed by standard hybridoma technology.² Human protein C mAbs 1575 and 1580 (HPC1575 and

HPC1580) were obtained by screening the ability of the generated mAbs for blocking the binding of FL-APC to EA.hy926 cells by Fluorescence-activated cell sorting (FACS).

Briefly, EA.hy926 cells were incubated with 50 nM FL-protein C and 100 nM various monoclonal antibodies against protein C in HBSS (Hanks' Balanced Salt Solution) buffer containing 0.5% BSA, 3 mM CaCl₂ and 0.6 mM MgCl₂ for 30 minute on ice, and then subjected to FACS analysis.

Human APC mAb 1573 (HAPC1573) was obtained by screening the binding ability of the generated mAbs to APC but not protein C with an ELISA assay. Briefly, 96 well MaxiSorp plate (NUNC) were coated with 5 µg/ml different mAbs in 15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6 buffer overnight at 4°C. The plate was washed with TTBS (TBS containing 0.05% Tween-20) containing 1 mM CaCl₂ (TTBS calcium buffer), blocked with 0.1% gelatin in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h, washed again and incubated with 100 ng/ml protein C or APC in TTBS calcium buffer for 1 h. Following wash, the plate was incubated with 2 µg/ml biotinylated HPC1580 for 1 h, washed again and incubated with 1 µg/ml streptavidin-alkaline phosphatase conjugate in TTBS calcium buffer for another hour. The endpoint absorbance at 405 nm was read on a Vmax microplate reader after final washing followed by addition of p-nitrophenyl phosphate liquid substrate (Sigma).

ELISA assay for APC in plasma

The assay was modified from the previously described ELISA assay for screening mAbs against APC. Briefly, the plate was coated with 5 µg/ml HAPC1573, blocked with TBS

containing 1X casein (Vector Lab) and washed with TTBS calcium buffer. Recombinant APC from 0-8 ng/ml in TTBS containing 10 mM benzamidine, 1 mM EDTA and 0.25X casein buffer (Dilution buffer) or 1:4 diluted human plasma was added to the wells and the plate was incubated for 1 h. After washing with TTBS calcium buffer, the plate was incubated with 1 µg/ml biotinylated HPC1575 in TTBS containing 10 mM benzamidine, 5 mM CaCl₂ and 0.25X casein buffer for 1 h. After washing, the plate was incubated with 0.5 µg/ml streptavidin-HRP in TTBS containing 10 mM benzamidine, 5 mM CaCl₂ and 0.25X casein buffer for another hour. Absorbance at 450 nm was recorded after color development with Ultra-TMB substrate (Pierce) and reaction termination with 0.5 M H₂SO₄.

APC anticoagulant activity assay

The effects of mAbs on APC anticoagulant activity in plasma was determined in a one-stage clotting assay initiated by the factor X coagulant protein (X-CP) from Russell's viper venom. Clotting times were measured using ST4 coagulation instrument (Diagnostica Stago). 50 µl of buffer with or without APC (6.4 µg/ml) or HAPC1573, HPC1575 and mouse IgG1 isotype mAb (160 µg/ml), 25 µl of phospholipids vesicles (80 µg/ml of 40% phosphatidylethanolamine, 20% phosphatidylserine and 40% phosphatidylcholine, w/v), 25 µl of X-CP, 50 µl of pooled normal human plasma and 50 µl of 25 mM CaCl₂ were used in this assay. Except the plasma, all reagents were in a buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5 and 0.1% BSA. The X-CP concentration was adjusted to give a clotting time of 31 seconds. Clotting was initiated by the addition of the calcium.

Thrombin-induced endothelial cell permeability

To assess the effects of mAbs on APC inhibition of thrombin induced endothelial cell permeability, the flux of Evans blue dye across an endothelial monolayer was measured as previously described.³ Briefly, EA.hy926 cells were seeded in transwells of 3 µm pore size and 12 mm diameter (Costar) for 3 days. The cells were incubated with 20 nM APC with or without 100 nM HAPC1573, HPC1575 or mouse IgG1 isotype mAb in DMEM containing 0.4% BSA for 3 h and then replaced with DMEM containing 0.4% BSA and 5 nM bovine thrombin for 10 min. The upper chamber was replaced with DMEM containing 0.4% BSA and 0.67 mg/ml Evans blue and the lower chamber was replaced with DMEM containing 0.4% BSA. The Evans blue medium in the lower chamber was measured for OD 650 after 2 min incubation.

Results

HAPC1573 detected only APC but not protein C in plasma by ELISA assay

HAPC1573 was screened out by an ELISA method, which only recognized APC but not protein C, from all monoclonal antibodies generated from mice immunized with human APC. Our data show that using HAPC1573 as first antibody and biotin labeled HPC1575 as second antibody, the sandwich ELISA method could detected spiked APC serial diluted from 8 ng/ml to 0.25 ng/ml in 1:4 diluted normal pooled plasma (NPP). The standard curve of spiked APC in diluted NPP was almost identical to the spiked APC in the dilution buffer which has no plasma proteins at all, indicating that there was no non-specific interaction between

plasma proteins including protein C and the coating mAb for APC, HAPC1573

(Supplemental Figure 2).

HAPC1573 blocks APC anticoagulant activity in plasma

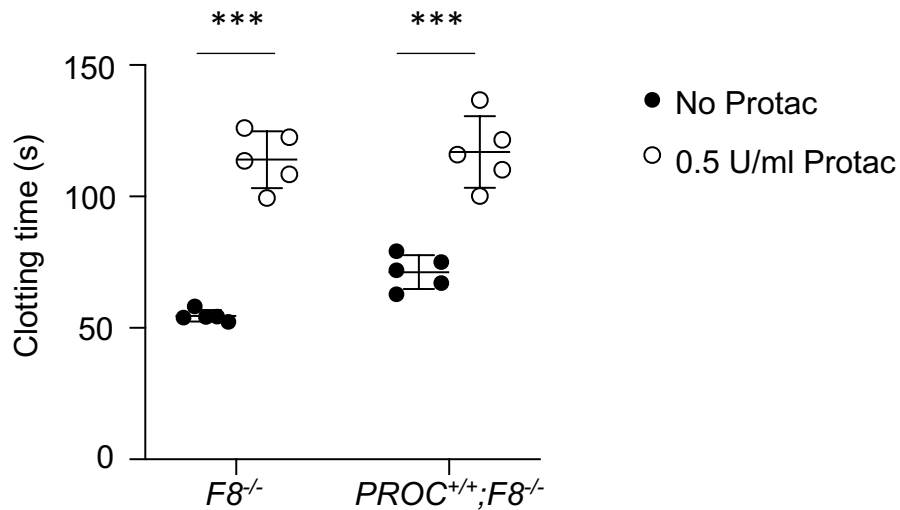
Supplemental Figure 3 showed that HAPC1573 almost completely diminished the prolongation effect of APC in factor Xa initiated one-stage plasma clotting assay, suggesting that the interaction of HAPC1573 and APC prevents APC from cleaving factor Va.

HAPC does not block APC cytoprotective activity on endothelium

The APC cytoprotective effects on endothelium *in vitro* was measured by APC inhibition of thrombin-induced permeability. HPC1575, but not HAPC1573, blocked this APC effect indicating that HAPC1573 would not affect APC signaling *in vitro* (Supplemental Figure 4).

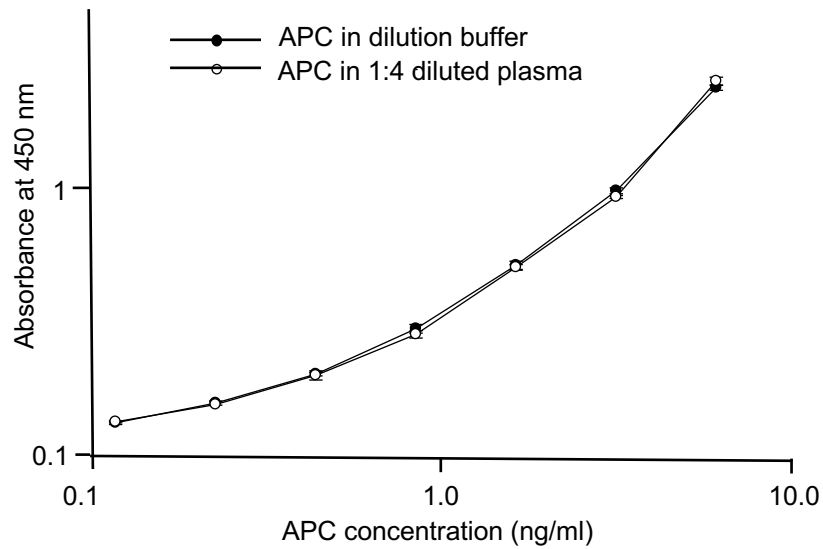
Supplemental Figures and Legends

Supplemental Fig. 1



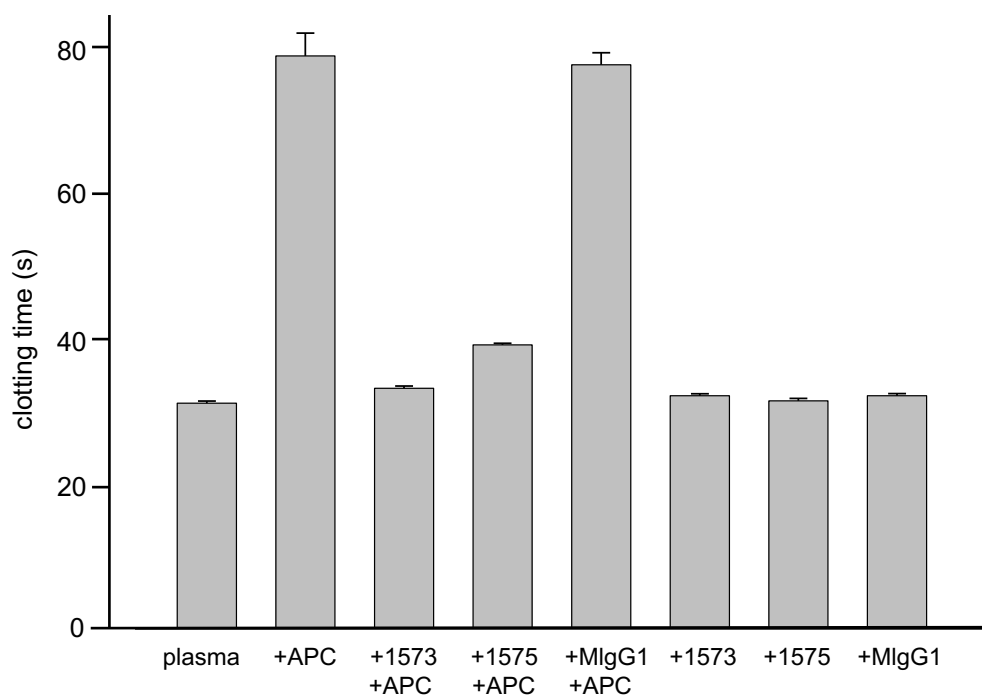
Supplemental Figure 1. Human protein C has similar anticoagulant activities in mouse plasma *in vitro*. Plasma samples from $F8^{-/-}$ mice and $PROC^{+/+};F8^{-/-}$ mice were diluted in two folds, and incubated with or without 0.5 U/ml of Protac, a protein C activator, for 15 min, and then $CaCl_2$ was added to trigger clotting formation. The clotting time was recorded by an automated analyzer. Data were expressed as mean \pm SD. $n = 5$ mice/group. ***, $P < 0.001$ (two-way ANOVA test).

Supplemental Fig. 2



Supplemental Figure 2. HAPC1573 detected only APC but not protein C in plasma. In the ELISA assay, the plate was coated with HAPC1573 and then spiked with APC of various concentrations (0-8 ng/ml) in 1:4 diluted pooled normal human plasma or in dilution buffer without any plasma protein. After washing, bound APC was detected by biotin labeled HPC1575, which recognize both APC and protein C. These assays were performed in duplicates and the errors were within 5%.

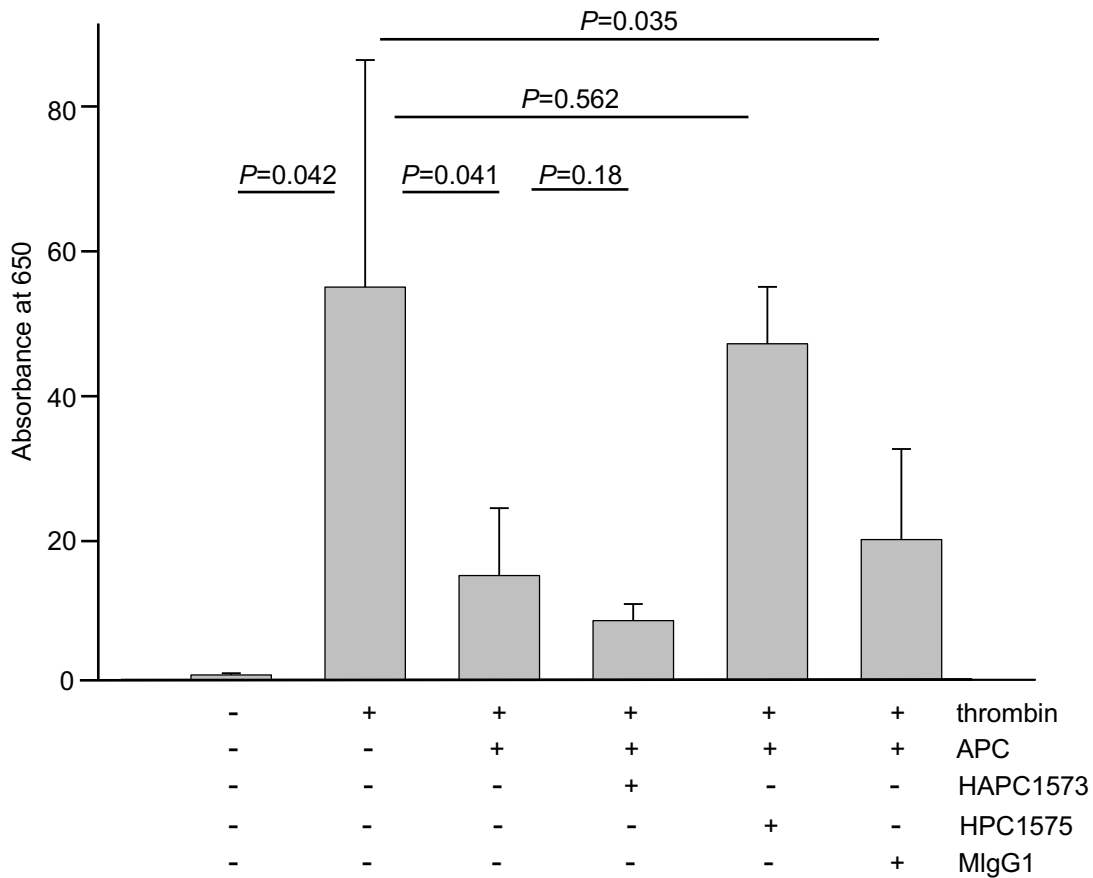
Supplemental Fig. 3



Supplemental Figure 3. Both HAPC1573 and HPC1575 blocked APC anticoagulant

activity. In this Russell's viper venom (which contains factor X activator) based one-stage clotting assays with normal human plasma, either APC alone, various antibodies alone, or combination of APC and one of the antibodies were added into the assay reactions. Clotting times were measured using ST4 coagulation instrument (Diagnostica Stago). The assays were performed in four replicates and the results shown are the means \pm SD. APC, 1.6 μ g/ml; HAPC1573, HPC1575 and mouse IgG1 isotype mAb, 40 μ g/ml.

Supplemental Fig. 4



Supplemental Figure 4. HPC1575 but not HAPC1573 blocked APC inhibition of

thrombin-induced endothelial permeability. In this assay, EA.hy926 cells seeded in

transwells were incubated with or without 20 nM APC, 100 nM HAPC1573, HPC1575 or

mouse IgG1 isotype mAb, as indicated by the plus and minus signs. Permeation of Evans

blue dye through the cells was induced by the addition of thrombin, and the endothelial

permeability was observed via tracking flux of the dye across the cell monolayer into cell

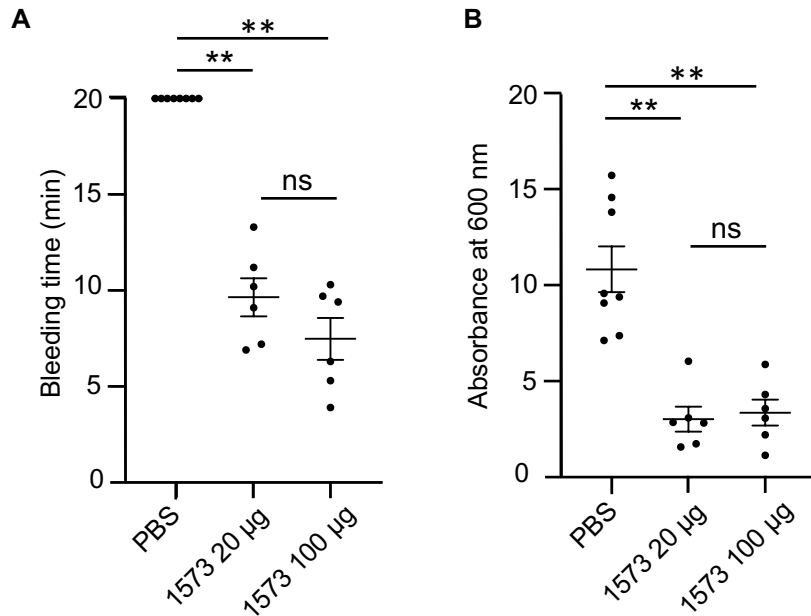
medium in another chamber. Absorbance at 650 nm of the medium was recorded. The results

shown are the means \pm SD performed in four replicates, and t-test was used to compare data

of the pairs indicated in the figure. Differences were considered statistically significant at P

value < 0.05 .

Supplemental Fig. 5



Supplemental Figure 5. Effects of 20 and 100 µg/mouse of HAPC1573 on tail bleeding time and the volume of lost blood of *PROC*^{+/+};*F8*^{-/-} mice. *PROC*^{+/+};*F8*^{-/-} mice were treated with either 20 or 100 µg/mouse of HAPC1573 30 min before the procedure. Pretreatment with PBS was used as a negative control. (A) Tail tip bleeding time was recorded for a maximum of 20 min to avoid mortality caused by over bleeding. (B) The tips of mouse tail were immersed in 15ml falcon tubes filled with saline. Hemolysis of the collected blood was performed and blood loss was quantified by recording the absorbance at 600 nm. Data were expressed as mean ± SD. n = 6 mice/group. ns, $P \geq 0.05$, **, $P < 0.01$ (one-way ANOVA test).

References:

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