Activated protein C anticoagulant activity is enhanced by skeletal muscle myosin

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Supplementary Appendix

Methods

Preparation and activation of Protein C and Factor V

Plasma-derived Protein C was purified as described (1) and activated to provide APC for FVa inactivation studies. Protein C was activated by thrombin that was coupled to Sepharose beads as suggested by the manufacturer (Pharmacia, Uppsala, Sweden). Thrombin beads were removed by centrifugation when a maximum of amidolytic activity of APC towards substrate S2366 (DiaPharma, West Chester, OH) was reached. The APC was stored at -80^{o}C in small aliquots. For experiments testing the effects of SkM on activation of protein C, recombinant wild type Protein C was used (2). Except as noted, FV was purified and activated with thrombin as described (3, 4). Thrombin was quenched with a 10% molar excess of hirudin. FVa was stored in small aliquots at -80^{o}C.

Other proteins and reagents

Human FXa, thrombin, prothrombin, and ProS were from Enzyme Research Laboratories (South Bend, IN). Rabbit skeletal muscle myosin (SkM) (95% homologous to human SkM) was from Cytoskeleton, Inc (Denver, CO) and was dialyzed at 7.8 µM and 4^{o}C into 0.6 M NaCl, 50 mM Tris-HCl, pH 7.4, and stored at -80^{o}C in small aliquots that were thawed immediately before a single use. SkM for some experiments was purchased as a solution in 50% glycerol and stored at -20^{o}C (Sigma, St. Louis, MO). No differences were noted between SkM from the two sources. Soluble thrombomodulin was from American Diagnostica/Sekisui (Greenwich, CT). The amidolytic substrate for APC was Pefachrome PCa (Pentapharm. Basel, Switzerland).

PL vesicles were prepared by sonication: 6 X 30 s pulses with 1 min rest in between, in an ice bath and under a flow of and N_2, and were composed of 75% phosphatidyl choline, 20% phosphatidyl ethanolamine and 5% phosphatidyl serine (Avanti Polar Lipids, Alabaster AL). When PL or SkM were used at nM concentrations, they were diluted into Hepes-buffered saline, 0.5% BSA, pH 7.4 (HBS) immediately before use from concentrated stock solutions.

Monoclonal antibody against FVa heavy chain (5146) was from Haematologic Technologies (Essex Junction, VT). Fatty acid-free bovine serum albumin (BSA) was from PAA Laboratories (Dartmouth, MA)

FVa activity assays (Prothrombinase assays)

Two stage assays were used to determine the effects of SkM on FVa inactivation by APC. FVa activity after various times of incubation with or without APC and SkM ± ProS in the first stage was measured in the second stage. In the first stage, 1-3.7 nM FVa was incubated in HBS containing 0.5% BSA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3, with or without APC (0.1-1 nM) and with or without ProS (20-100 nM) in the presence of SkM or PL vesicles, in wells of v-well polypropylene plates (Thermo-Fisher, Rochester, NY). After specified incubation times, for the second stage, small aliquots (usually ≤ 5% of the reaction mixtures) were transferred to other wells and mixed with other reagents for standard prothrombinase assays. For second stage assays, microplate wells contained 0.01-0.02 nM of the transferred FVa from the first stage mixture, 1 nM FXa and 25 µM PL. Prothrombin was added and mixed to give 1.0 µM (final). Subaliquots were removed at 30 s intervals over 2 min and added to flat-well plates (Bacti polystyrene plates, Thermo-Fisher) containing quenching buffer (10 mM EDTA, 0.05 M Tris-HCl, 0.1 M NaCl2, 0.02% NaN3, pH 8.0. Chromogenic substrate for thrombin (pefachrome TH, Pentapharm/Centerchem, Norwalk, CT) was added to a final concentration of 0.2 mM and the linear rate of change of absorbance at 405 nm was measured. For controls, the rate of change of absorbance gave the thrombin concentration for FVa mixtures without APC added (control) and this was taken as 100% activity. Subaliquots taken for control assays produced ~1 nM thrombin/min.

Immunoblotting analysis

Incubation mixtures of FVa with and without SkM, with and without APC, and with and without ProS were prepared as above, except that BSA was decreased to 0.01%. Aliquots of various reaction mixtures were heated for 10 min at 80^{o}C in Licor LDS sample buffer containing 10 mM EDTA, treated with 10 mM iodoacetamide, then electrophoresed using 4-15% Criterion denaturing gels (Bio-Rad, Richmond, CA). Proteins were transferred to Immobilon-FL membranes (Millipore, Ballerica, MA), blocked with Odyssey blocker-TBS (Li-Cor, Lincoln, NE), and developed with 2 µg/ml monoclonal mouse anti-FVa heavy chain antibodies in Odyssey blocker plus 2 mM $CaCl_2$ + 0.1% Tween-20, followed by infra-red dye-labeled secondary antibodies (donkey anti-mouse IgG, Li-Cor) diluted 1:8,000.

Plasma Coagulation assays with SkM and APC

Plasma assays for APC anticoagulant activity in the presence of SkM used an Amelung KC 4 coagulometer (Sigma Diagnostics, St Louis MO), as follows: *FXa One-Stage Clotting Assays:* A mixture of 50 µL of FX-depleted plasma (FXdP, Enzyme Research Labs), 50 µL of 50 nM FXa (Enzyme Research Labs), and 50 µL of dilutions of SkM (200, 150, 100, 50, 25, 12.5, 0 nM) was incubated for 180 sec at 37°C. Clotting was initiated by adding 50 ul 70 nM of APC in diluting buffer containing of 30 mM $CaCl_2$. *Kaolin Clotting Time (KCT) Assays*: All reagents were diluted in 20 mM Tris, 100 mM

NaCl pH 7.4 containing 1 mg/mL BSA. Clotting assays were performed by incubating a mixture of 50 µL FV-depleted plasma (FVdP, Enzyme Research Labs), 5 µL of 22 nM FVa (Hematologic Technologies), 50 µL of dilutions of SkM (200, 150, 100, 50, 25, 12.5, 0 nM), and 50 µL of Kaolin (activator vial from Diagnostica Stago C.K. Prest5 kit, Taverny, France) for 180 sec at 37°C. The clotting times were recorded after adding 50 µL of 70 nM APC in diluting buffer containing 30 mM $CaCl_2$.

Statistical analyses

Error bars on graphs represent standard error of the mean (S.E.M.) of 2-5 experiments performed on different days. Unpaired student's t tests or ANOVA for multiple comparisons were performed when appropriate, and comparison results were considered significant if p values were ≤ 0.05 (GraphPad Prizm, San Diego, CA).

Supplementary References

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