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Quantitative analysis of thrombomodulin-mediated conversion of Protein C to APC: translation from *in vitro* to *in vivo*

Ronald Carnemolla^{1,2}, Kruti Rajan Patel^{1,2}, Sergei Zaitsev^{1,2}, Douglas B. Cines³, Charles T. Esmon⁴, and Vladimir R. Muzykantov^{1,2}

¹Department of Pharmacology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

²Program in Targeted Therapeutics of the Institute for Translational Medicine and Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

³Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

⁴Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

Abstract

Thrombomodulin-bound thrombin cleaves protein C (PC) zymogen in blood plasma producing activated protein C (APC), which exerts anti-coagulant, anti-inflammatory, anti-apoptotic and CNS-protective effects. Recombinant APC and thrombomodulin (TM) are both in clinical studies for management of acute conditions including sepsis. Methods that permit accurate measurement of APC in plasma are needed for clinical monitoring and mechanistic studies in animal models. However, the two existing methods require either long incubation periods with substrate resulting in high background or they also recognize protein C inhibitor (PCI) complexed with APC (APC:PCI), which convolutes analysis of the amount of APC generated. Here we describe a robust quantitative *in vivo* assay that measures APC generation at both low levels of human protein C seen in chronic inflammatory disease and at physiological levels that shows a >99% fit with *in vitro* data.

1. Introduction

The endothelial surface glycoprotein thrombomodulin (TM), binds thrombin and facilitates thrombin-induced cleavage of the plasma zymogen protein C into an active protease, activated protein C (APC), which inhibits coagulation, inflammation, apoptosis and vascular edema (1–2). Recombinant APC and TM are in extensive translational and clinical studies as prospective therapeutic interventions for treatment of sepsis and diverse acute vascular disorders (3–6). Experimental models to study TM/APC in animals, including genetically modified mice, provide an opportunity to identify potential targets and to permit pre-clinical testing. To do so, precise monitoring of TM/APC activity is needed to correlate levels of

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Address for correspondence: Ronald Carnemolla, Perelman School of Medicine, University of Pennsylvania, Translational Research Center, Building 421, TRC 10–178, 3400 Civic Center Boulevard, Philadelphia, PA 19104, Phone: 215–746–2337, FAX: 215–573–2236, rcarne@mail.med.upenn.edu.

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APC and TM protein (both endogenous and therapeutic) with functional readouts and experimental outcomes.

However, measuring APC levels in mouse plasma has proven challenging. Two assays are currently available, each with its own limitations. The first detects a complex formed between APC and protein C inhibitor (PCI), and therefore does not detect free APC, the most important functional component (7). The second is an ELISA that uses a monoclonal antibody (mAb 1587) to capture both APC and protein C zymogen followed by measuring bound enzymatic activity using an amidolytic assay (8). However, protein C zymogen (present at high levels in plasma) competes with APC for binding to the capturing mAb, greatly reducing test sensitivity. As a result, measuring enzymatic activity of bound APC takes 16–24 hrs and is constrained by high background and imprecision.

To address this gap, we took advantage of an antibody specific for human APC (mAb 1555) (9). This antibody was used to measure APC in subjects with sepsis (10) and in competition from excess human protein C zymogen (9), in both cases yielding very high specificity. Here we examine the ability of mouse thrombomodulin-thrombin complexes to generate human APC *in vitro* and in mice. This approach generated a >99% fit between APC protein levels *in vitro* and *in vivo* and may thereby provide a useful tool to investigate the TM/APC system and its physiological and pathological regulation in murine models.

2. Methods

2.1. Materials

C57BL6J 6–8 week old male mice were purchased from Jackson Laboratories. Human protein C was kindly supplied by Dr. Sriram Krishnaswamy at the University of Pennsylvania. Human APC was purchased from Haemotologic Technologies (Essex Junction, VT, USA), Spectrazyme PCa from American Diagnostica, bovine thrombin from GE Amersham Biosciences, recombinant Hirudin from EMD Chemicals, benzamidine HCl hydrate and bovine serum albumin type V from Fisher Scientific, heparin from porcine intestinal mucosa from Sigma, 96-well Costar (cat# 3595) plates for *in vitro* experiments and 8-well EIA/RIA Corning strips (cat# 2590) for *in vivo* experiments from Fisher Scientific. Mouse monoclonal antibodies against human APC were used as described (9).

2.2. Generation of soluble thrombomodulin (sTM): cDNA encoding the extracellular

domain of TM (Leu¹⁷-Ser⁵¹⁷) was amplified from whole C57BL6J mouse lung cDNA. sTM was constructed with a triple-FLAG tag at the 3' end and purified by anti-FLAG affinity chromatography. Resulting protein fractions were concentrated using a 10kDa MW cutoff. sTM was quantitated using a nanodrop, with a MW of 56.7kDa and an extinction coefficient of 1.09.

2.3. Quantitation of APC levels *in vitro*

2.3a. Human protein C dose response *in vitro*—Human protein C (32.25, 64.5 and 322.5nM) was incubated with sTM (29.3nM) and bovine thrombin (7.36nM) in 0.1 mL PBS (w/Ca²⁺), for 10 min in a 1.7mL microcentrifuge tube. A 25μl aliquot was removed and added to 25μl recombinant hirudin [0.25U/μl] to quench thrombin activity in each well within a 96-well plate. A 50μl solution of Spectrazyme (2μM) was added and the OD at 405nm was at 60 min. Values for the 60 min time point from three independent experiments were averaged, N=3 ± SD. The molarities of human protein C and bovine thrombin were calculated based on MWs of 62 and 36.7kDa, respectively.

2.3b. Thrombomodulin dose response *in vitro*—Human protein C (64.5nM) was incubated with sTM (0.293, 2.93, 29.3 and 1470nM) and bovine thrombin (7.36nM) in 0.1mL PBS (w/Ca²⁺) for 10 min in a 1.7mL microcentrifuge tube. A 25μl aliquot was removed and placed in each well within a 96-well plate, 25μl recombinant hirudin [0.25U/μl] and a 50μL solution of Spectrazyme (2μM) were added, and the OD was measured at 405nm at 60 min. Values for independent experiments at the 60 min time point were averaged, N=3 ± SD.

2.4. Quantitation of APC levels in mouse plasma

2.4a. Human protein C dose response *in vivo*—We assumed an average blood volume of 1.5mL for a 25g mouse. sTM (29.3nM) and bovine thrombin (7.36nM) were pre-incubated for 10 min and injected into the jugular vein of C57BL6J mice. Immediately thereafter, human protein C (32.25, 64.5 or 322.5nM) was injected into the contralateral jugular vein. Ten min later, blood was collected from the inferior vena cava in 3.8% sodium citrate and benzamidine HCl (v/v 2:1), with 100μl of the sodium citrate mix added per 600μl of collected blood. Blood was spun at 1500xg for 5 min. Plasma was collected and flash frozen in liquid nitrogen and stored at -80°C until use.

2.4b. Thrombomodulin dose response *in vivo*—sTM (0.293, 2.93, 29.3 and 1470nM) and bovine thrombin (7.36nM) were pre-incubated for 10 min before injection into the jugular vein of C57BL6J mice. Immediately thereafter, human protein C (64.5nM) was injected into the contralateral jugular vein. Ten min later, blood was collected from the inferior vena cava in 3.8% sodium citrate and benzamidine HCl (v/v 2:1). 100μl of the sodium citrate:benzamidine HCl mix was added per 600μl of blood. Plasma was prepared as above.

2.4c. Human APC detection by antibody based ELISA—Anti-human APC mAb 1555 (5μg/mL) in 20mM Hepes, 150NaCl, 5mM CaCl₂, pH7.5 was allowed to bind overnight at 4°C to high-binding EIA/RIA 8-well strips. Wells were washed twice with 20mM Hepes, 150NaCl, 5mM CaCl₂, 0.05% Tween-20, pH7.5 and blocked with 20mM Hepes, 150NaCl, 5mM CaCl₂, 1% BSA, pH7.5 for 1 hr at room temperature. Wells were then washed 2 times for 5 min each. Human APC standards [0–3200ng/mL] were prepared in mouse plasma diluted 1:4 in 20mM Hepes, 150NaCl, 15mM CaCl₂, 0.1% BSA, 2U/mL heparin, 20mM benzamidine HCl, pH7.5 (100μl/well). Human APC standards spiked with human APC antibody [50μg/mL] to lower background (antibody non-specificity) were prepared in parallel. Plasma samples from mice given various doses of human PC or sTM were prepared identically to standards. Standards and samples were incubated at room temperature for 1 hr, washed 4 times for 5 min each. A 100μl solution of 1μM Spectrazyme (diluted 1:10 in coating buffer) was added to each well and the OD at 405nm was measured every min for 2 hrs. Raw values for human APC were subtracted from human APC values obtained in the presence of excess anti-human APC mAb [50μg/mL]. APC values were then extrapolated from the linear equation based on the standard curve. Human APC values [ng/mL] are reported as the average of N=3–4 ± SD.

3. Results and Discussion

We describe the development and characterization of an assay to measure the ability of thrombomodulin and thrombin to generate human APC *in vitro* and *in vivo* in mice. First, we measured human APC generation *in vitro*. The amount of APC generated by thrombin-TM complexes was proportional to PC concentration within the range of 32.5–322.5nM and was not affected by mouse plasma, which contains an excess of mouse protein C zymogen (Figures 1 and 2A).

Capitalizing on this finding, we measured human APC generated by TM-thrombin complexes in mice pre-injected with sub-, equal- and supra-physiological concentrations of human PC (Figure 2B and Table 1). Using human PC concentrations that are equal to physiological concentrations of endogenous mouse PC, the amount of human APC we measured is up to 1.5 times higher than the levels of endogenous mouse APC detected with a mouse-specific APC ELISA.

We next compared the quantitative relationship of human APC measured *in vitro* and *in vivo* (Figure 2C). Comparison of the measurements gave a fit that exceeded 99%. This capacity to measure the conversion of sub-physiological PC to APC is especially useful in states of chronic inflammation, where protein C levels can decrease by 50% or more. Of course, substituting murine PC zymogen with its human analogue using a mouse as a living test tube is not the same as measuring murine APC *in vivo*. However, this approach provides the opportunity to monitor thrombomodulin function in pre-clinical studies in this animal species.

Thrombin associated with TM converts PC to APC up 1000 times faster than thrombin alone. Therefore, APC production by thrombin may be used to analyze this cofactor activity of TM, such as injected recombinant TM. To address this directly, we measured APC generation *in vitro* and *in vivo* by exogenous TM. To do so, we incubated or injected soluble TM together with thrombin and a fixed amount of human PC. TM increased production of human APC *in vivo* up to 60-fold higher than baseline endogenous TM and exogenous thrombin controls (Figure 3, Tables 1–2). Using 29.3 or 1470nM exogenous TM, the increase in circulating human APC after injection of TM was similar to the amount measured using a mouse-specific APC ELISA (85ng/mL). Again, the relationship between APC generation *in vitro* to *in vivo* exceeded >99% (Figure 3C).

In summary, we describe a novel method that enabled us to measure the APC generating capacity of TM *in vitro* and *in vivo* in mice. Our results suggest existing methods of measuring mouse APC by ELISA may underestimate the impact of thrombomodulin, in part because the antibody (mAb 1587) recognizes mouse PC and APC comparably. Our experimental design demonstrates >99% correlation between APC detection *in vitro* and *in vivo* with thrombin-mediated conversion of human PC to APC dependence upon both PC and TM concentration. Use of this assay for monitoring APC-producing function of exogenous and endogenous TM in mouse models including genetic mutant animals used as a “live test tube” for injection of human PC zymogen, will facilitate basic and preclinical studies of TM/APC system.

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Figure 1. Mouse Protein C or other plasma components do not bind to Human APC antibody Human APC standards 0–160ng/mL were run in parallel in human or mouse plasma and a curve was fitted for standards in mouse plasma (open circles) or human plasma (closed circles), $R^2 = 0.991$.

Fig. 2A **Fig. 2B** **Fig. 2C**

Figure 2. Conversion of human PC to APC is dependent upon PC concentration *in vitro* and *in vivo*

A) Sub-, equi- or supraphysiological concentrations of human PC (32.25, 64.5 and 322.5nM) were incubated with sTM (29.3nM) and thrombin (7.36nM) (open circles) versus thrombin alone (closed circles) for 10 min. Hirudin was added, followed by Spectrazyme. The concentration of APC (ng/mL) was extrapolated from a standard curve generated with human APC. $N=3 \pm SD$. **B)** sTM (29.3nM) and thrombin (7.36nM) (open circles) versus thrombin alone (closed circles) were pre-incubated for 10 min before injection into one jugular vein followed by injection of sub-, equi- or supraphysiological concentrations of human PC (32.25, 64.5 and 322.5nM) into the contralateral vein. Plasma was analyzed for human APC using a sandwich based ELISA for human APC. APC concentrations were measured as above. $N=3 \pm SD$. **C)** Fitted curve for normalized *in vitro* (open circles) and *in vivo* (closed circles) APC values for the dependence on PC concentration, $R^2 = 0.9908$.

Fig. 3A **Fig. 3B**

Fig. 3C

Figure 3. Human PC to APC conversion is dependent upon TM concentration *in vitro* and *in vivo*
A) A physiological concentration of human PC (64.5nM) was incubated with increasing concentrations of sTM (0.293, 2.93, 29.3 and 1470nM) and thrombin (closed circles) for 10 min (7.36nM). Hirudin and Spectrazyme were added as above. APC concentrations were determined as above. $N=3 \pm SD$. **B)** Increasing concentrations of sTM (0.293, 2.93, 29.3 and 1470nM) and thrombin (7.36nM) (closed circles) were preincubated for 10 min prior to injection as in Fig. 2 followed by human PC (64.5nM). Plasma APC was measured using a sandwich based ELISA for human APC. APC concentrations were measured as above. $N=3 \pm SD$. **C)** Fitted curve for normalized *in vitro* (open circles) and *in vivo* (closed circles) APC values for the dependence on TM concentration, $R^2 = 0.9943$.

Table 1
Conversion of human PC to APC is dependent on PC concentration *in vivo*

Percentage of human PC converted to APC based on data from experiments shown in Fig. 1B). APC concentrations were determined as above. N=3 ± SD.

sTM concentration [nM]	Starting PC concentration [ng/mL]	Resulting APC Concentration [ng/mL]	% conversion of PC to APC
0	2000	0	0
29.3	2000	54.0	2.7
0	4000	1.52	0.039
29.3	4000	88.73	2.218
0	20000	15.52	0.077
29.3	20000	289.53	1.447

Table 2
Conversion of human PC to APC is dependent on TM concentration *in vivo*

Percentage of human PC converted to APC based on data from Fig. 2B. APC concentrations were determined as above. N=3 $\pm$ SD.

sTM concentration [nM]	Starting PC concentration [ng/mL]	Resulting APC Concentration [ng/mL]	% conversion of PC to APC
0	4000	1.56	0.039
0.293	4000	11.69	0.292
2.93	4000	50.08	1.252
29.3	4000	71.88	1.797
1470	4000	84.08	2.102