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Antithrombotic activity of protein S infused without activated protein C in a baboon thrombosis model

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Summary

Protein S (ProS) is an essential plasma protein that enhances the anticoagulant activity of activated protein C (APC). *In vitro*, purified native human Zn²⁺-containing ProS also exerts direct anticoagulant activity by inhibiting prothrombinase and extrinsic FXase activities independently of APC. We investigated antithrombotic effects of ProS infused without APC in a baboon shunt model of thrombogenesis that employs a device consisting of arterial and venous shear flow segments. In *in vitro* experiments, the Zn²⁺-containing human ProS used for the studies displayed >10-fold higher prothrombinase inhibitory activity and anticoagulant activity in tissue factor-stimulated plasma, and 4-fold higher inhibition of the intrinsic pathway than the Zn²⁺-deficient ProS used. In the thrombosis model, ProS (33 µg/min for 1 hr) or saline was infused locally; platelet and fibrin deposition in the shunt were measured over 2 hr. During experiments performed at 50 mL/min blood flow, Zn²⁺-containing ProS inhibited platelet deposition 73–96% in arterial-type flow segments and 90–99% in venous-type flow segments; Zn²⁺-deficient ProS inhibited platelet deposition 52% in arterial-type flow segments and 65–73% in venous-type flow segments. At 100 mL/min blood flow rate, Zn²⁺-containing ProS inhibited platelet deposition by 39% and 73% in the respective segments; Zn²⁺-deficient ProS inhibited platelet deposition by 5% and 0% in the respective segments. Zn²⁺-containing ProS suppressed fibrin deposition by 67–90%. Systemic APC-independent ProS activity was significantly increased and thrombin-antithrombin complex levels were significantly decreased after infusion of ProS. Thus, infused human Zn²⁺-containing ProS is antithrombotic in primates, and may have therapeutic potential even in protein C-deficient human patients.

Keywords

anticoagulants; platelets; protein S; thrombosis model

Introduction

Protein S (ProS) is an essential vitamin K-dependent plasma anticoagulant. Homozygous deficiency of ProS usually leads to life-threatening purpura fulminans at birth (1), and heterozygous deficiency of ProS leads to increased risk of venous thrombosis (2). Heterozygous deficiency may also be associated with increased risk of arterial thrombosis at

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a young age (3). Genetic ablation of ProS in a mouse model resulted in an embryonically lethal phenotype with severe coagulopathy (4). ProS is best known as a cofactor for APC during proteolytic inactivation of Factor (F)Va and FVIIIa (5), but ProS also has direct anticoagulant activity (sometimes referred to as ProS-direct) that is independent of APC. Purified ProS binds and inhibits FXa, FVa, FVIIIa and tissue factor and it directly inhibits both prothrombinase and extrinsic FXase activities (6–11). ProS-direct exhibits widely varying potencies that we found to be due to loss of previously unrecognized intramolecular Zn^{2+} in ProS, with subsequent change in conformation, during some purification procedures that involve quaternary anion exchange resins (12). Zn^{2+} -containing ProS, but not Zn^{2+} -deficient ProS, exhibits good ProS-direct *in vitro*, and ProS in plasma exhibits ProS-direct that is similar to that of ProS-depleted plasma reconstituted with Zn^{2+} -containing ProS (10, 12, 13). Zn^{2+} -containing ProS inhibits extrinsic FXase independently of tissue factor pathway inhibitor (TFPI) (10), while Zn^{2+} -deficient ProS inhibits extrinsic FXase only in conjunction with TFPI (14).

APC-independent anticoagulant activity of ProS was demonstrated in plasmas from heterozygous ProS-deficient mice, because the plasmas exhibited shorter base clot times and greater thrombin generation after a prothrombinase stimulus than did wild-type mouse plasmas (4). We hypothesized that the APC-independent anticoagulant activity of ProS is a real biologic activity and that ProS infused without APC would have antithrombotic effects in a primate thrombosis model. To test this hypothesis, we employed a well-characterized, quantifiable baboon thrombosis model that was used in preclinical tests of APC (15–17). We infused either Zn^{2+} -containing ProS or Zn^{2+} -deficient ProS in the absence of APC. Since APC was used, though discontinued, as an anticoagulant and anti-inflammatory drug to treat severe sepsis (18), it is pertinent to determine if ProS could have therapeutic potential.

Materials and Methods

Antibodies and reagents

Sheep anti-protein C antiserum was a gift from Dr. Hans Peter Schwarz of Immuno/Baxter, Vienna, Austria, and antibodies that block APC activity were immunoaffinity-purified from the serum. Rabbit anti-TFPI was a kind gift of Drs. Sam Rapaport, Bonnie Warn-Cramer, and Vijaya Rao, formerly of the University of California, San Diego. Monoclonal antibodies against human ProS were prepared as described (7). Phospholipids were from Avanti, Alabaster, AL, USA, and phospholipid vesicles (80% phosphatidyl choline, 20% phosphatidyl serine) were prepared by sonication (7). FXa and prothrombin were from Enzyme Research Laboratories, South Bend, IN, USA. FV was prepared and activated as described (7). Plasma-derived Zn^{2+} -containing ProS was barium adsorbed from citrated plasma, eluted with ammonium sulfate, treated with 4.4% polyethylene glycol to remove ProS in complex with C4b-binding protein, and immunoaffinity-purified on a column of monoclonal antibody S7 coupled to Sepharose (10). Recombinant ProS (rProS) was immunoaffinity-purified from concentrated conditioned media. Zn^{2+} -deficient rProS (rProS Q) was a kind gift from Dr. Brian Grinnell of Eli Lilly, and was prepared using MonoQ-Sepharose chromatography (19). Thrombin-antithrombin complexes were measured with a kit from Boehringer-Ingelheim, Petersburg, VA, USA. Rabbit anti-human ProS was obtained from Dako, Carpinteria, CA, USA. Thrombin fluorogenic substrate ZGGR-aminomethyl-coumarin was from Bachem, Torrance, CA, USA, and pefaTH chromogenic substrate for thrombin was from Centerchem, Norwalk, CT, USA. Alexin APTT reagent was from Sigma, St. Louis, MO, USA.

Prothrombinase assays

FVa (20 pM), 0.5 nM FXa and 25 μ M phospholipid vesicles (80% phosphatidylcholine/20% phosphatidylserine) were preincubated 10 min with or without ProS in the presence of 0.5% BSA, 5 mM CaCl_2 , in Hepes-buffered saline, pH 7.4 (HBS: 50 mM Hepes, 100 mM NaCl) (6). Prothrombin was added to 0.6 μ M and aliquots were taken at 30 s intervals and quenched in 20 mM EDTA. Thrombin chromogenic substrate (0.2 mM final concentration) was added to measure the rate of thrombin generation under each condition, using a kinetic plate reader at 405 nm.

Measurement of thrombin generation in human and baboon plasma

Two types of thrombin generation assays in plasma were used to show the direct anticoagulant activity of ProS in the intrinsic, and in the extrinsic, coagulation pathways, and to monitor the activity of two types of purified ProS in plasma. First, a 2-stage clotting assay previously described (13) was modified as a dilute APTT assay that reflects the intrinsic coagulation pathway. Briefly, 15 μ l of control normal human plasma (CNP) or ProS-depleted plasma (ProSdP) was pretreated for 8 min with sufficient thrombin inhibitor I-2581 to prevent thrombin feedback activation of coagulation in stage 1 (2.4 μ M in stage 1), with and without neutralizing antibodies against APC, ProS, and/or TFPI, in a volume of 25 μ l. 15 μ l of HBS, 5 mM CaCl_2 , 1 mM MgCl_2 , 0.5% BSA, pH 7.4, containing 25 μ M phospholipid vesicles was added, followed by 3.2 min incubation with 10 μ l of APTT reagent diluted 1:12 in the same buffer. Plasma ProS could thus interact with procoagulant components in the presence of CaCl_2 without thrombin feedback activation of coagulation. In stage 2, the effect of the thrombin inhibitor was diluted by addition of 200 μ l of the same buffer containing phospholipids. Aliquots were removed over time and quenched in 20 mM EDTA. Thrombin chromogenic substrate was added to the quenched mixtures and the rate of thrombin generation was assessed in a kinetic plate reader at 405 nm. Thrombin generation in various samples was compared at times when thrombin generation in ProSdP was significantly greater than in CNP.

A second type of plasma thrombin generation assay was triggered via the extrinsic coagulation pathway by dilute tissue factor and was used in prior reports to show the direct anticoagulant activity of ProS in human plasma (12). It was applied to baboon plasma for the current studies. Briefly, 45 μ l of baboon plasma was preincubated for 8 min with sufficient affinity-purified antibodies against APC (120 μ g/mL) to neutralize twice the maximum APC that could be formed from plasma protein C, with and without 300 μ g/mL neutralizing polyclonal antibodies against human ProS in a final volume of 50 μ L. In some cases, human ProS was added to 80 nM in the plasma. A stimulus of dilute tissue factor, CaCl_2 , phospholipid vesicles, and thrombin fluorogenic substrate was added to final concentrations of 0.3 pM, 12 mM, 10 μ M, and 0.5 mM, respectively, in a final volume of 100 μ L. Under these conditions corn trypsin inhibitor had almost no effect, indicating that the intrinsic pathway of coagulation was a minor contributor to thrombin generation (10). Thrombin generation was followed by fluorescence over time and the lag time was taken as the time to a change in fluorescence of 100 units min^{-1} .

Baboon thrombosis model

In this model, thrombus forms within an exteriorized arterio-venous shunt in response to a thrombogenic surface that is placed under controlled flow and geometry (14–17, 20). Systemic anticoagulation and animal sacrifice are not required. Blood flow is subject to normal blood recirculation and filtration systems, as well as normal pathways for activation or inhibition of coagulation enzymes, platelets and other blood cells.

A chronic silicone rubber shunt was surgically implanted between the femoral vein and artery of each animal. Animals were pre-infused with autologous ^{111}In -labeled platelets (1 mCi) and, in selected experiments, with ^{125}I -labeled baboon fibrinogen (5 μCi). At the beginning of each experiment, a thrombogenic device was inserted into the arteriovenous shunt. Each device consisted of a proximal infusion site for ProS or saline, followed by a thrombogenic Dacron vascular graft segment, followed by a chamber of expanded diameter that produces venous-type blood recirculation and stasis, and then a region where an extended tail thrombus was propagated.

Upon initiating blood flow through the graft-chamber device, saline or ProS in saline was infused upstream from the Dacron graft for 1 hr along the inner surface of the shunt while platelet deposition was quantified by gamma camera imaging of the graft. In this local infusion method, ProS was infused through a cuff made from ringed thin-walled expanded polytetrafluoroethylene (WL Gore Associates, Flagstaff, AZ, USA) placed 1 cm upstream from the Dacron segment. Assembly of the device was achieved by connecting sections using silicone rubber medical adhesive (Type A, Corning), resulting in a smooth isodiametric transition between the surfaces. Imaging continued for an additional hour after cessation of infusions. Blood flow was controlled with a clamp and monitored with a flow meter. At the end of the experiment, device segments were removed from the shunt and ^{111}In activity was allowed to decay before ^{125}I -fibrin deposition was measured in a gamma counter.

At 30 min intervals during the infusion, blood samples were taken from the proximal silicone rubber shunt (~28 inches proximal from the Dacron segment. Plasma was prepared and frozen at -80°C for various assays.

All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon Health and Science University in accordance with US Federal guidelines. Occasional anxiety in the animals was relieved with low dose ketamine (≤ 2 mg/hr, i.m.).

ProS dosing and spacing of experiments

Ten thrombosis experiments were performed in four male baboons (*Papio anubus*), 9–11 kg each. The infusion rate was 6 mL/hr (using a Harvard pump) and the infused ProS concentration was 0.333 mg/mL (2 mg total, 0.2 mg/kg). The dose of ProS infused was chosen to nearly double the concentration of free ProS in baboon plasma, if it was administered systemically rather than locally, and ignoring clearance. (A 10 kg baboon has ~500 mL blood, ~275 mL of plasma. $2 \text{ mg ProS} / 275 \text{ mL} = 7.3 \mu\text{g ProS/mL}$, compared to 10 $\mu\text{g/mL}$ free ProS in circulation in humans, similar to the concentration in baboons.)

Control (saline infusion) experiments were performed in the same animal as one or two ProS infusion experiments. Each experiment was spaced from another by one day, with one exception where the control was performed 5 days before ProS infusion. Each infusion of rProS Q was performed in the same animal as a control infusion and a ProS S7 infusion. The sequences were as follows. Animal 1: control, Zn^{2+} -ProS S7, Zn^{2+} -deficient ProS Q; Animal 2: Zn^{2+} -ProS S7, control, Zn^{2+} -deficient ProS Q; Animal 3: Zn^{2+} -ProS S7, control; Animal 4: control, Zn^{2+} -ProS S7.

Statistics

For comparison of thrombin-antithrombin complexes or lag times in thrombin generation assays, two-tailed t tests were used. For comparison of platelet deposition under different conditions, one-way ANOVA for repeated measures and Newman-Keuls post-test for multiple comparisons were applied, using Graph Pad software, San Diego, CA, USA. Values of $p \leq 0.05$ were considered significant.

Results

***In vitro* characterization of purified ProS and demonstration of APC-independent ProS activity in plasma**

Zn²⁺-containing human plasma-derived ProS from a number of affinity-purified preparations was pooled for baboon thrombosis studies. In standardized prothrombinase assays, various preparations of ProS inhibited 50% (IC₅₀) of thrombin generation at ProS concentrations of 6.5–8.9 µg/mL (87–119 nM). Seven affinity-purified plasma-derived ProS preparations were analyzed for Zn²⁺-content, and contained a mean of 1.4±0.6 atoms of Zn²⁺ per molecule (12). Several preparations of affinity-purified human recombinant ProS (rProS) were pooled and had an IC₅₀ in prothrombinase assays of 8.0 µg/mL (107 nM). These immunoaffinity-purified rProS preparations had a mean of 0.9±0.3 atoms of Zn²⁺ per molecule. Dose-response of this rProS in prothrombinase assays is shown (Fig. 1A). Neutralizing antibodies against TFPI did not affect inhibition of either prothrombinase or extrinsic FXase by Zn²⁺-containing ProS, thus the preparations did not contain detectable TFPI (10). rProS that was purified using Mono Q chromatography in the presence of EDTA, then in the presence of Ca²⁺, was Zn²⁺-deficient, contained 0.15 atoms of Zn²⁺ per molecule, and had low ProS-direct (Fig. 1A). In previous studies, Zn²⁺-deficient ProS had less than one-tenth the APC-independent anticoagulant activity of Zn²⁺-containing ProS in thrombin generation assays triggered by dilute tissue factor or by FXa (11, 12).

To show an APC-independent effect of the ProS via the intrinsic pathway in plasma, we developed a dilute APTT assay (see also ref. 8). Thrombin was generated more rapidly in ProSdP than in CNP that contains ProS (Fig. 1B, diamonds versus closed circles). This assay had almost no response to neutralizing TFPI or protein C antibodies in either CNP or ProSdP (Fig. 1B, squares and triangles). Yet, the assay was highly responsive to neutralizing monoclonal antibody against ProS, even in the presence of neutralizing antibodies against protein C and TFPI (Fig. 1B, open circles). Thrombin generation in CNP treated with the anti-ProS antibody was very similar to that in ProSdP. Thus, at least some modes of ProS-direct in plasma are TFPI- and APC-independent. Zn²⁺-containing ProS (ProS S7) added at the level of free ProS in plasma could approximately reconstitute ProSdP, so that the initial rate of thrombin formation diminished to nearly the same levels observed in CNP (Fig. 1C) (21). Zn²⁺-deficient ProS caused ~1/4 as much diminution of the initial rate of thrombin generation. However, the rate was not quite significantly different from the rate in ProSdP.

To establish an APC-independent effect of plasma ProS via the extrinsic pathway in baboon plasma, as previously done in human plasma (10, 12), we used dilute tissue factor as a clotting stimulus in a thrombin generation assay. In this type of assay, the lag time until threshold thrombin generation is the parameter most sensitive to ProS (10). When normal baboon plasma was preincubated for 8 min with neutralizing antibodies against human ProS, the lag time decreased from 6.24 min to 4.95 min, and peak thrombin generation increased (Fig. 1D, triangles compared to squares). It is uncertain whether these anti-human ProS antibodies could totally neutralize baboon ProS, so we may not have observed the maximum possible changes due to neutralization of ProS. Addition of 80 nM Zn²⁺-containing human ProS to normal baboon plasma caused an increase in lag time to 8.11 min and lower peak thrombin generation (X's), while addition of 80 nM Zn²⁺-deficient human ProS had no detectable effect (open circles). Neutralizing antibodies against APC were included in the assays to ensure that the differences reflected the APC-independent anticoagulant activity of ProS and not the APC cofactor activity of ProS. Thus, ProS has direct anticoagulant activity when either the intrinsic or extrinsic coagulation pathways prevail, although its activity may be partly dependent on TFPI in the extrinsic pathway (10). We cannot completely exclude that a tiny amount of APC was generated despite the excess neutralizing anti-APC antibodies used, but overall, these results suggest that baboon plasma ProS exerts APC-

independent anticoagulant activity and that exogenous human ProS infused without APC should be able to exert antithrombotic activity in baboons.

ProS inhibition of platelet deposition in the thrombosis model at a blood flow rate of 50 mL/min

Saline with or without 2 mg of Zn^{2+} -containing plasma-derived ProS immunopurified on S7-Sepharose (ProS S7) was infused for 1 hr at 6 mL/hr along the lumen of the arterio-venous shunt, upstream of the three regions of a thrombogenic device in which platelet deposition was measured over time, at a blood flow rate of 50 mL/min. Compared to the saline control, ProS S7 suppressed platelet deposition to various degrees, depending on the segment being monitored and the time elapsing after termination of the infusion (Fig. 2). Platelet deposition was significantly suppressed during ProS infusion in the thrombogenic Dacron segment that simulated thrombus generation under arterial flow conditions (Fig. 2A), and was nearly completely suppressed in the chamber of larger diameter that simulated venous flow recirculation and stasis (Fig. 2B) as well as in the region of the propagating tail thrombus (Fig. 2C). When ProS infusion was terminated after 1 h, platelet deposition gradually increased in all segments but remained depressed compared to the controls for an additional hr.

Zn^{2+} -containing recombinant (r)ProS purified on S7-Sepharose (rProS S7) was similarly compared to saline control and to Zn^{2+} -deficient rProS purified on MonoQ Sepharose (rProS Q) (Fig. 3A–C). Zn^{2+} -containing rProS S7 nearly completely prevented platelet deposition during the 1 hr infusion in all three segments, and continued to significantly suppress platelet deposition for 1 hr after termination of infusion in the chamber and in the propagating tail region. Zn^{2+} -deficient rProS was significantly less effective at suppression of platelet deposition in all three segments over the 2 hr experiment but was nearly as effective as rProS S7 in the propagating tail thrombus during the 1 hr infusion period.

ProS inhibition of platelet deposition at a flow rate of 100 mL/min

For experiments performed at a higher shunt blood flow rate of 100 mL/min, the saline control occluded after 55 min of infusion (Fig. 4) and no further control data points were collected. For this reason, the means $\pm$ standard deviations for historical controls were plotted with this series for the Dacron segment and the chamber. (No historical data are available for the propagating tail thrombus region.) Affinity-purified Zn^{2+} -containing ProS S7 was significantly effective in suppressing platelet deposition at the higher flow rate, though less so than at the flow rate of 50 mL/min. Suppression of platelet deposition by affinity-purified ProS S7 was less in the Dacron segment than in the chamber region of venous-type flow (Fig. 4, comparing panels A and B). Zn^{2+} -deficient rProS that was purified using MonoQ chromatography had little, if any, ability to suppress platelet deposition in any of the segments at the higher shunt flow rate of 100 mL/min.

Results of the series at flow rates of 50 mL/min and 100 mL/min are summarized in Table 1. At a flow rate of 50 mL/min, inhibition of platelet deposition at 1 hr by Zn^{2+} -containing immunoaffinity-purified ProS and rProS was 73–99% in the various segments, and was greater than inhibition by Zn^{2+} -deficient MonoQ-purified rProS. At a flow rate of 100 mL/min, affinity-purified ProS inhibited platelet deposition somewhat less than at a 50 mL/min flow rate, while Zn^{2+} -deficient MonoQ-purified rProS had almost no ability to inhibit platelet deposition.

Deposition of fibrin and formation of thrombin-antithrombin complexes

Following 50 mL/min experiments, thrombogenic devices were removed, and ^{125}I -fibrin deposited (from preinfused ^{125}I -baboon fibrinogen) was measured after decay of the ^{111}In -

label in the platelets, and fibrin deposited in each segment was calculated. Terminal fibrin deposition was suppressed by affinity-purified Zn^{2+} -containing ProS as compared to the saline control. Suppression of fibrin deposition was greater in the chamber (90%) than in the Dacron segment (67%).

Blood was taken upstream of the thrombogenic devices into citrate prior to insertion of the thrombogenic graft, after the 1 hr infusions, and after the additional 1 hr in which platelet deposition was measured. Plasma was prepared and thrombin-antithrombin (TAT) complexes were measured (Fig. 5A). Compared to saline infusion, infusion of plasma-derived Zn^{2+} -containing ProS depressed TAT formation by 67% at the end of the 1 hr infusion, and TAT remained depressed by 43% at the end of the 2 hr observation period. Thus, ProS had a significant antithrombotic effect on both platelet deposition and TAT formation.

APC-independent activity of ProS in baboon plasma before and after infusions

Plasma samples from blood taken upstream during and after infusion of saline or Zn^{2+} -containing plasma-derived ProS were subjected to thrombin generation assays with a dilute tissue factor stimulus in the presence of neutralizing antibodies against APC to assess the APC-independent activity of plasma ProS (Fig. 5B). The lag time before thrombin generation is the parameter most sensitive to the APC-independent anticoagulant activity of plasma ProS in this protocol (Fig. 1D and ref. 11). Setting the initial lag time of plasma on day 1 prior to insertion of the graft (6.5 min) to 100%, saline infusion after graft insertion resulted in a significant shortening of the lag time. On plasma samples from day 2 ProS infusion, lag times were significantly prolonged at the end of the 1 hr ProS infusions. Thus, the systemic APC-independent anticoagulant activity of ProS was significantly increased during local ProS infusions ($p \leq 0.01$). Compared to saline controls at 2 hr, the lag times with ProS infusion were also increased at the end of the 2 hr observation period, but these differences were not statistically significant. This dose of ProS, chosen to raise the concentration of free ProS in the baboon plasma by ~73%, raised the APC-independent anticoagulant activity of ProS significantly in the systemic circulation.

APC cofactor activity of ProS was also measured in these plasma samples by a published method (22). Samples taken 60 min after ProS infusion had 1.18-fold higher APC cofactor activity than the “pre” samples ($p=0.048$); samples taken 120 min after ProS infusion did not show an increase in APC cofactor activity of ProS.

Discussion

Human ProS infused without exogenous APC was antithrombotic in this baboon thrombosis model, though we cannot say at this point what proportion of the antithrombotic activity was APC-independent. The ProS dose of 0.2 mg/kg-hr was somewhat less than the doses of APC (0.25–1.1 mg/kg-hr) used in this model in previous trials (15). At low blood flow, Zn^{2+} -containing rProS that was immunoaffinity-purified was more antithrombotic than Zn^{2+} -deficient rProS purified using MonoQ-Sepharose. At high blood flow, Zn^{2+} -deficient rProS had little antithrombotic effect, while Zn^{2+} -containing ProS remained significantly antithrombotic. A disparity between the two types of ProS in APC-independent anticoagulant activity *in vitro* was also noted in clotting assays and prothrombinase assays. We recently discovered that intramolecular Zn^{2+} is essential for efficient ProS inhibition of prothrombinase activity, inhibition of extrinsic FXase activity in the absence of TFPI, and high affinity binding to FXa and tissue factor (10,12). Zn^{2+} content does not affect the APC cofactor activity of ProS *in vitro* (12), but it did affect the antithrombotic effect of ProS in the baboon thrombosis model.

Zn^{2+} is known to inhibit the amidolytic activity but not anticoagulant activity of APC and to enhance protein C and APC binding to endothelial cell protein C receptor (23, 24). Zn^{2+} induces conformational change in protein C and APC; Zn^{2+} -containing ProS also has a different conformation than Zn^{2+} -deficient ProS (12). It is important to note that exogenous Zn^{2+} can affect APC and protein C functions, while exogenous Zn^{2+} cannot affect ProS activity. Zn^{2+} appears to be incorporated within the ProS molecule during synthesis/secretion, but can be lost during certain purification procedures. No exogenous Zn^{2+} was infused with ProS in the experiments presented here.

ProS was more antithrombotic at lower blood flow rates that simulate venous flow, in keeping with the clear association of low ProS levels in humans with increased risk of venous thrombosis, while there are few reports of an association of low ProS levels with risk of arterial thrombosis, which takes place at higher blood flow. Similarly, ProS was more antithrombotic in the lower flow, lower shear rate chamber of the baboon model than in the higher flow, higher shear rate Dacron segment. Nevertheless, ProS clearly inhibited platelet deposition, which is commonly thought of in association with arterial thrombosis. The mechanism(s) of inhibition of platelet deposition by ProS in this model remains to be clarified, but may be due to inhibition of thrombin generation and subsequent inhibition of platelet stimulation by thrombin.

ProS infusion also inhibited fibrin deposition and formation of thrombin-antithrombin complexes in the thrombosis model. It caused a significant increase in direct anticoagulant activity measured as the lag time for thrombin generation after a stimulus of dilute tissue factor in baboon plasma from blood taken upstream from the infusion site. Systemic APC cofactor activity of ProS also increased somewhat 60 min after ProS infusion, thus the antithrombotic effect of ProS could have been due to both direct anticoagulant activity and APC cofactor activity of ProS. APC levels in baboons with control grafts are 21–32 ng/mL compared to 12 ng/mL in resting baboons and a mean of 250 ng/mL APC generated in baboons during low-dose thrombin infusion (16, 25).

Clear statistical differences were observed for all affinity-purified plasma-derived ProS or affinity-purified rProS versus control experiments. At a blood flow of 50 mL/min, Zn^{2+} -deficient rProS purified using MonoQ also differed statistically from the saline control in all three segments that were monitored, but at a flow of 100 mL/min, this Zn^{2+} -deficient rProS did not differ significantly from the control. Numerous prior experiments show that this model is fairly reproducible, as evidenced by the error bars for historical controls in Fig. 4 and for replicates in Fig. 2.

Zn^{2+} -containing ProS is a multifunctional anticoagulant protein, inhibiting prothrombinase, extrinsic FXase, and the intrinsic pathway as well as serving as a cofactor to APC. Zn^{2+} -deficient ProS is reported to enhance the interaction of TFPI with FXa (14, 26), and both Zn^{2+} -deficient and Zn^{2+} -containing ProS bind tightly to TFPI ($K_{d_{app}}=21$ nM) (11). Thus, there may be both TFPI-independent and TFPI-dependent modes of ProS-direct in plasma. This remains to be fully clarified in future thrombosis model experiments, since plasma ProS purified by mild methods that do not include either anion exchange or affinity purification appears to be mostly Zn^{2+} -containing (12). Zn^{2+} -deficient ProS had modest activity in dilute APTT assays, and in the infusion experiments presented here, suggesting that part of its antithrombotic effect in the infusion experiments may have been via inhibition of the intrinsic pathway and/or enhancement of TFPI activity. More detailed mechanistic studies in a thrombosis model should be performed in the future, and it would be informative to infuse ProS, mini-ProS, and the SHBG region of ProS in the presence and absence of neutralizing antibodies against TFPI and APC.

The major conclusion is that during significant thrombotic challenge such as used here, exogenous Zn^{2+} -containing ProS has therapeutic promise. ProS may have some potential advantages as an antithrombotic therapeutic over other anticoagulants, including possibly the use of APC, since it is more stable than an enzyme, has a longer half-life *in vivo*, and it is more abundant if a plasma source is used. Future model studies should include ProS in combination with other anticoagulants, such as APC, for possibly greater efficacy than is achieved by either anticoagulant alone. Some of the therapeutic effect of APC in sepsis is thought to be due to its anti-inflammatory effects (18). ProS also has anti-inflammatory effects as a ligand for the receptor tyrosine kinase Mer that are being explored (27, 28), and it would be worth testing whether increasing ProS levels in sepsis has the potential to generate outcome benefits. While most marketed anticoagulants have dose-limiting antihemostatic effects, it appears that increasing free ProS levels to nearly twice normal does not adversely affect hemostasis, although this question remains to be fully evaluated in appropriate models.

Information table

What is known about this topic?

- Protein S (ProS) is an essential anticoagulant plasma protein that serves *in vitro* as a cofactor to activated protein C (APC), and in some cases, to tissue factor pathway inhibitor (TFPI).
- *In vitro*, ProS also has direct anticoagulant activity, independent of APC and TFPI, that depends on retention of intramolecular Zn^{2+} during purification procedures.

What does this paper add?

- ProS infused without APC is antithrombotic in a baboon thrombosis model.
- Zn^{2+} -containing ProS is more antithrombotic than Zn^{2+} -deficient ProS.
- ProS is a potential antithrombotic therapeutic for human primates that could be infused alone, or in combination with other antithrombotic drugs.

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Figure 1. *In vitro* demonstration of direct anticoagulant activity of ProS

(A) Comparison of the ability of rProS prepared by two different methods to inhibit prothrombinase activity in purified component assays described in Methods. (B) Thrombin generation in a dilute APTT assay in control normal human plasma (CNP) versus ProS-depleted plasma (ProSdP). The assay was performed in the presence and absence of neutralizing antibodies (α) against APC, TFPI, and/or ProS, as indicated. See Methods for details. (C) Reconstitution of ProSdP in the dilute APTT assay with either Zn²⁺-deficient ProS purified using MonoQ chromatography (ProS Q) or Zn²⁺-containing ProS (ProS S7), each at 10 μ g/mL (130 nM) in ProSdP. Incubation time with APTT reagent was shortened by 1 min compared to panel B, and thrombin generation was measured at 7 min. (D)

Thrombin generation in normal baboon plasma (NBP) stimulated with dilute tissue factor. Plasma was preincubated 8 min with a mixture of neutralizing rabbit anti-human ProS antibodies and monoclonal antibody S4 (triangles), or with either Zn²⁺-containing ProS (ProS S7, X's) or Zn²⁺-deficient ProS purified using MonoQ chromatography (ProS Q, open circles), each at 80 nM, or with HBS-0.5% BSA (closed squares). A dilute tissue factor stimulus and fluorogenic thrombin substrate were added as described in Methods. The rate of thrombin generation was monitored as fluorescence units min⁻¹. The lag time until 100 fluorescence units min⁻¹ is indicated.

Figure 2. Platelet deposition in the baboon thrombosis model at 50 mL/min blood flow in the presence and absence of Zn^{2+} -containing plasma-derived ProS
 Platelet deposition was quantified by gamma camera imaging in the three segments of the graft during 1 hr infusion of ProS or saline control, and for 1 hr after the infusion was stopped. (A) Platelet deposition in the thrombogenic Dacron segment. (B) Platelet deposition in the chamber. (C) Platelet deposition in the propagating tail thrombus. Circles, saline infusion; squares, infusion of immunoaffinity-purified plasma-derived ProS (ProS S7). A matched set of 4 experiments was compared by paired t test. In all 3 segments, platelet deposition with ProS infusion differed significantly from that of saline controls ($p < 0.0001$).

Figure 3. Platelet deposition at 50 mL/min blood flow in the absence or presence of recombinant (r)ProS purified by two different methods

Zn^{2+} -containing rProS S7 (diamonds) was immunoaffinity-purified using monoclonal antibody S7 coupled to Sepharose, and Zn^{2+} -deficient rProS Q (triangles) was purified using MonoQ Sepharose with a calcium gradient. By one-way ANOVA analysis for repeated measurements, platelet deposition in the 3 experiments differed significantly in all 3 segments ($p < 0.0001$). By Newman-Keuls multiple comparisons post-test, values in all three segments were $p < 0.001$ for each rProS versus the control. Platelet deposition with rProS S7 versus rProS Q differed significantly: $p < 0.001$ in the Dacron segment; $p < 0.05$ in the chamber; and $p < 0.01$ in the propagating tail segment.

Figure 4. Platelet deposition in the baboon thrombosis model at 100 mL/min blood flow
Experiments were similar to those in Figs. 2 and 3 except that blood flow was adjusted to 100 mL/min. Closed circles, saline control infusion; open circles, means and standard deviations for historical controls; triangles, infusion of rProS purified on MonoQ Sepharose (rProS Q); squares, infusion of immunoaffinity-purified plasma-derived ProS (ProS S7). Because the control graft occluded after 55 min of saline infusion, historical controls were also plotted. By one-way ANOVA analysis for repeated measurements, platelet deposition in the groups in all 3 segments varied significantly ($p \leq 0.006$) during the infusion (5–55 min). By Newman-Keuls multiple comparisons post-test, platelet deposition with ProS S7 infusion differed significantly from control infusion $p < 0.001$ in the Dacron segment and in

the chamber, but platelet deposition with rProS Q did not ($p>0.05$). ProS S7 differed significantly from rProS Q ($p<0.01$).

Figure 5. Effect of ProS infusion on thrombin-antithrombin (TAT) complex formation and thrombin generation in baboon plasma

Blood was collected upstream from the graft at several time points on each of two experiments conducted at a blood flow rate of 50 mL/hr, and plasma was prepared. (A) TAT complexes in the plasma of the baboon were measured during saline infusion on day 1 and during ProS infusion on day 2 in the same baboon. Asterisks indicate a significant difference as compared to the analogous time point in the saline control ($p \leq 0.007$). "Pre" denotes the baseline level immediately before the experiment. (B) Thrombin generation assays were performed on baboon plasma after a stimulus of dilute tissue factor, CaCl_2 , and phospholipids, in the presence of neutralizing antibodies against APC. The lag time until

threshold thrombin generation (100 fluorescence units/min) was the parameter most sensitive to ProS. There was a statistically significant difference in the lag time for the saline control after 60 min infusion compared to “pre”. There was also a statistically significant difference in the control versus ProS infusion after 60 min ($p \leq 0.01$). Mean lag time for day 1 “pre” plasma was 6.5 min. Each bar in A and B represents two or more assays performed on different dates.

Table 1

Inhibition of platelet deposition in the baboon thrombosis model after 1 hr ProS infusion.

		% Inhibition, 50 mL/min		% Inhibition, 100 mL/min	
		rProS Q	rProS S7	ProS S7	ProS S7
Dacron segment	52	96	73-99	5	39
Chamber	65	98	95-99	0	73
Propagating tail	73	90	92-95	0	N.D.

Each ProS was infused at 2 mg/hr for 1 hr. Percent inhibition is relative to platelet deposition at the 1 hr time point in the saline controls (taken as 0% inhibition) in the same animal performed one day before or after the ProS infusion. The ranges for ProS S7 at 50 mL/min include two experiments. rProS Q, rProS purified using MonoQ Sepharose; rProS S7, rProS immunoaffinity-purified using monoclonal antibody S7; ProS S7, immunoaffinity-purified plasma-derived ProS. N.D., not determined.