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Platelet Factor 4 Impairs the Anticoagulant Activity of Activated Protein C*

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Platelet factor 4 (PF4) is an abundant platelet α-granule chemokine released following platelet activation. PF4 interacts with thrombomodulin and the γ-carboxyglutamic acid (Gla) domain of protein C, thereby enhancing activated protein C (APC) generation by the thrombin-thrombomodulin complex. However, the protein C Gla domain not only mediates protein C activation *in vivo*, but also plays a critical role in modulating the diverse functional properties of APC once generated. In this study we demonstrate that PF4 significantly inhibits APC anticoagulant activity. PF4 inhibited both protein S-dependent APC anticoagulant function in plasma and protein S-dependent factor Va (FVa) proteolysis 3- to 5-fold, demonstrating that PF4 impairs protein S cofactor enhancement of APC anticoagulant function. Using recombinant factor Va variants FVa-R506Q/R679Q and FVa-R306Q/R679Q, PF4 was shown to impair APC proteolysis of FVa at position Arg^{306} by 3-fold both in the presence and absence of protein S. These data suggest that PF4 contributes to the poorly understood APC resistance phenotype associated with activated platelets. Finally, despite PF4 binding to the APC Gla domain, we show that APC in the presence of PF4 retains its ability to initiate PAR-1-mediated cytoprotective signaling. In summary, we propose that PF4 acts as a critical regulator of APC generation, but also differentially targets APC toward cytoprotective, rather than anticoagulant function at sites of vascular injury with concurrent platelet activation.

Protein C is a vitamin K-dependent glycoprotein zymogen, which is activated by the thrombin-thrombomodulin complex on the surface of endothelial cells (for review, see Refs. 1–3). Protein C activation is enhanced by protein C binding to its receptor, the endothelial cell protein C receptor (EPCR)[2] (4, 5). Activated protein C (APC) down-regulates the coagulation cascade by inactivation of procoagulant cofactors, factor Va (FVa) and factor VIIIa (FVIIIa) (6, 7), both of which are required for thrombin generation. The clinical importance of APC anticoagulant function is well established. Homozygous protein C deficiency causes purpura fulminans in neonates (8), whereas heterozygous deficiency is associated with significantly increased risk of venous thrombosis (9). Proteolysis of FVa and FVIIIa by APC is enhanced >20-fold in the presence of protein S, which acts as a cofactor for APC (10). Consequently, inherited or acquired protein S deficiency is also associated with significantly increased risk of thrombosis (11, 12). Recent studies have demonstrated that APC bound to EPCR via its Gla domain can also initiate protease-activated receptor 1 (PAR-1)-mediated anti-inflammatory and anti-apoptotic signaling in endothelial cells (13, 14). Furthermore, the cytoprotective properties of APC are of therapeutic benefit to patients with severe sepsis syndrome (15).

FVa inactivation by APC occurs due to limited proteolytic cleavage of FVa at positions Arg^{506} and Arg^{306} (16). Previous *in vitro* studies have demonstrated that FVa inactivation by APC occurs rapidly in the presence of negatively charged phospholipid vesicles (16, 17). In contrast, APC-mediated FVa inactivation is markedly inhibited in the presence of activated platelets (17, 18). The one or more mechanisms through which platelet-mediated APC resistance occurs are unclear. However, previous work has indicated that platelet-mediated inhibition of APC anticoagulant function is enhanced by an unidentified protein component of the platelet secretory granules released following platelet activation (19).

Accumulating evidence suggests that platelet factor 4 (PF4) may play an important role in regulating protein C activation (20). PF4 is synthesized in megakaryocytes, and subsequently stored in platelet α-granules (20). In contrast to other cationic proteins, PF4 enhances APC generation up to 25-fold on endothelial cell surfaces (21, 22). This specific enhancement has been attributed to a PF4-mediated increase in the affinity of the thrombin-thrombomodulin complex for protein C, resulting in a 30-fold decreased K_m for protein C activation (22). Moreover, subsequent studies demonstrated that enhancement is mediated through the interaction between PF4 and the protein C Gla

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[2] The abbreviations used are: EPCR, endothelial cell protein C receptor; APC, activated protein C; FVa, factor Va; FVIIIa, factor VIIIa; FXa, factor Xa; PAR-1, protease-activated receptor 1; PF4, platelet factor 4; ETP, endogenous thrombin potential; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; BSA, bovine serum albumin; Gla, γ-carboxyglutamic acid.

domain (23). The protein C/APC Gla domain also plays critical roles in regulating both APC anticoagulant and cytoprotective signaling properties. However, the functional consequences of the interaction between the APC Gla domain and PF4 in relation to APC anticoagulant, anti-inflammatory, and anti-apoptotic functions have not previously been defined.

In this study, we demonstrate that PF4, in contrast to its role in protein C activation, attenuates APC anticoagulant activity in plasma. Furthermore, PF4 inhibits the ability of protein S to function as a cofactor for APC. Finally, despite PF4 binding to the protein C/APC Gla domain, we also demonstrate that the APC Gla domain interaction with EPCR is preserved, such that APC in the presence of PF4 retains its ability to initiate PAR-1-mediated cytoprotective signaling. Consequently, our findings support a novel physiological role for PF4 in modulating APC function.

EXPERIMENTAL PROCEDURES

Materials—Human PF4 was purchased from both Hematologic Technologies Inc. (Essex Junction, VT) and Enzyme Research Laboratories (South Bend, IN). Human purified APC, thrombin, and protein S were purchased from Hematologic Technologies Inc. Recombinant FVa variants FVa-R506Q/R679Q and FVa-R306Q/R679Q were generated and characterized as previously described (24). Deficient plasmas and chromogenic substrates BIOPHEN CS 21(66) and BIOPHEN CS-01(38) for APC and thrombin, respectively, were purchased from Hyphen-Biomed (Neuville-Sur-Oise, France). Thrombin generation assay reagents (PPP reagent, fluorogenic substrate, and thrombin calibration standard) were all purchased from Thrombinoscope BV (Maastricht, The Netherlands). Evans Blue dye, bovine serum albumin, and staurosporine were bought from Sigma. Pre-designed primers for reverse transcription-PCR experiments were from Applied Biosystems (Foster City, CA).

Determination of APC Anticoagulant Activity in the Presence of PF4 in Plasma—The anticoagulant function of APC in the presence of PF4 in normal, platelet-poor, pooled plasma was assessed using a Fluoroskan Ascent Plate Reader (Thermo Lab System, Helsinki, Finland) in combination with Thrombinoscope software (Thrombinoscope BV), as previously described (25). 80 μl of protein C-deficient plasma was incubated with 20 μl of PPP Reagent containing 5 pM tissue factor and 4 μM phospholipids (PC/PS/PE, 60%/20%/20%) in the presence or absence of APC (1.25–10 nM, all final concentrations). Thrombin generation was initiated by automatic dispensation of 2.5 mM fluorogenic thrombin substrate (Z-Gly-Gly-Arg-amidomethyl coumarin·HCl) and 100 mM $CaCl_2$ into each well (final concentrations, Z-Gly-Gly-Arg-amidomethyl coumarin·HCl, 0.42 mM, and $CaCl_2$, 16.67 mM). Thrombin generation was determined using a thrombin calibration standard. Measurements were taken at 20-s intervals for 60 min, or until thrombin generation was complete. The endogenous thrombin potential (ETP) of each reaction was then calculated. Experiments were performed in triplicate, and data were reported as mean ETP ± S.E.

Protein S Enhancement of APC Anticoagulant Activity in Protein S-deficient Plasma—APC anticoagulant activity in protein S-deficient plasma in the presence of PF4 was determined using a similar assay to that described above. Briefly, protein S-deficient plasma was incubated with APC (10 nM) in the presence of plasma-purified protein S (6.25–100 nM). Thrombin generation was initiated and assessed using Thrombinoscope software as described previously. All experiments were performed in triplicate and data plotted as mean ETP ± S.E.

Determination of Protein S-independent and Protein S-dependent APC-mediated FVa Proteolysis—FVa degradation by APC in the absence of protein S was assessed as previously described (26). Recombinant FVa (0.8 nM, final concentration) was incubated with phospholipid vesicles (PS/PE/PC, 10%/20%/70%, 25 μM final concentration) in the presence or absence of PF4 (1 μM, final concentration) in HNBSACa buffer (25 mM Hepes, 150 mM NaCl, pH 7.7, with 5 mg/ml BSA, and 5 mM $CaCl_2$). APC (1.65 nM, final concentration) was added to initiate FVa inactivation, and aliquots were drawn at set time intervals over a 20-min time course. FVa inactivation was stopped by 1/25 dilution in ice-cold HNBSACa. The FVa activity was measured at each time point using a prothrombinase assay containing 5 nM FXa, 0.5 μM prothrombin, and phospholipid vesicles (PC/PS, 90%/10%, 50 μM final concentration) for 2 min, then stopped with 40-fold dilution in ice-cold EDTA buffer (50 mM Tris-HCl, 100 mM NaCl, 20 mM EDTA, 1% polyethylene glycol 6000, pH 7.9). The amount of thrombin formed was measured using a thrombin-specific chromogenic substrate BIOPHEN CS-01(38). The residual FVa activity was determined by comparison with the FVa activity observed prior to APC incubation. The plotted values represent the mean of at least three individual experiments ± S.E. Protein S-dependent FVa proteolysis was measured at protein S concentrations in the presence or absence of PF4. Human protein S (2.5–25 nM) was incubated with 0.8 nM APC, 8 nM FVa, and 75 μM phospholipid vesicles (PC/PS/PE, 60%/20%/20%) in 40 mM Tris-HCl, pH 7.4, 140 mM NaCl, 3 mM $CaCl_2$, 0.3% (w/v) bovine serum albumin (0.2 nM APC, 2 nM FVa, and 19 μM phospholipid vesicles, final concentrations) for 2 min at 37 °C. A 2-μl aliquot was added to 0.3 nM FXa, 1.5 μM prothrombin, and 75 μM phospholipid vesicles (0.1 nM FXa, 0.5 μM prothrombin, and 25 μM phospholipid vesicles, final concentrations) at 37 °C for 3 min. 5 μl of ice-cold 250 mM EDTA stopped the reaction. The rate of thrombin substrate cleavage was then measured as before.

Calculation of Kinetic Rate Constants for APC-mediated Cleavage at Arg^{506} and Arg^{306} of FVa—To determine the pseudo-first order rate constants for APC-mediated cleavage of FVa inactivation sites Arg^{506} and Arg^{306}, inactivation of FVa-R306Q/R679Q and FVa-R506Q/R679Q was assessed over time, and curves were fitted to the data according to a previously determined equation (27). As each variant only possesses one cleavage site, the equation was modified to reflect this, as previously described (24). FVa degradation curves for FVa-R306Q/R679Q were fitted to Equation 1,

$$Va_t = Va_0 \cdot e^{-(k_{506}) \cdot t} + B \cdot Va_0 \cdot (1 - e^{-(k_{506}) \cdot t}) \qquad \text{(Eq. 1)}$$

where Va_t is the FVa cofactor activity determined at time point t, Va_0 is the FVa cofactor activity derived prior to incubation with APC, B is the remaining procoagulant activity of FVa

cleaved at amino acid position 506, and k_{506} is the rate constant for cleavage at position 506.

For determination of the rate constant for Arg^{306} cleavage, Equation 2 was used to fit the APC-mediated FVa degradation curve for FVa-R506Q/R679Q,

$$Va_t = Va_0 \cdot e^{-(k_{306}) \cdot t} + C \cdot Va_0 \cdot (1 - e^{-(k_{306}) \cdot t}) \quad \text{(Eq. 2)}$$

where C is the remaining procoagulant activity of FVa cleaved at position 306, and k_{306} is the rate constant for cleavage at position 306.

Measurement of Endothelial Cell Barrier Protection by APC—Endothelial cell barrier permeability was determined as described previously (28). Briefly, EAhy926 cells (kind gift of Dr. C. Edgell, University of North Carolina, Chapel Hill, NC) were grown to confluence on polycarbonate membrane Transwells (Costar, 3-μm pore size, 12-mm diameter) and incubated with 20 nM APC in the presence or absence of 1 μM PF4. After 3 h, the cells were treated with thrombin in serum-free media for 10 min. The cells were washed and incubated with 0.67 mg/ml Evans Blue with 4% bovine serum albumin (BSA). Changes in endothelial cell barrier permeability were determined by following the increase in absorbance at 650 nm in the outer chamber over time due to the transmigration of Evans Blue-BSA. Experiments were performed in triplicate and plotted as the mean ± S.E.

Determination of APC-mediated Protection of Apoptotic Endothelial Cells—Confluent EAhy926 cells in 6-well plates were pre-treated with APC ± PF4 for 17 h. EAhy926 cell apoptosis was induced by staurosporine (20 μM) treatment for 4 h. Cells were then trypsinized and RNA-extracted using an RNeasy Mini Kit (Qiagen). Reverse transcription was carried out (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) then Real Time PCR was performed using *Bax* (Hs00180269_m1), *Bcl-2* (Hs00153350_m1), and *β-actin* (Hs99999903_m1) TaqMan® Gene Expression Assays (Applied Biosystems) in a Applied Biosystems 7500 Real Time PCR system. Experiments were performed in triplicate and plotted as mean *Bax/Bcl-2* ratio ± S.E.

RESULTS

PF4 impairs APC Anticoagulant Function in Plasma—PF4 interacts with the protein C Gla domain, thereby significantly enhancing APC generation on endothelial cell surfaces (21, 22). However, the Gla domain also plays a critical role in regulating APC anticoagulant function. Preliminary experiments using an APC-specific chromogenic assay indicated that APC amidolytic activity was unaltered by the presence of PF4 concentrations up to 1 μM (data not shown). Consequently, calibrated automated thrombography was used to assess the impact of PF4 upon APC attenuation of tissue factor-initiated thrombin generation in protein C-deficient plasma (25). APC alone reduced thrombin generation (Fig. 1*A*). Calculation of the area under the thrombin generation curve (ETP) indicated that 10 nM APC reduced thrombin generation to 9 ± 4% of the original ETP prior to APC incubation (Fig. 1*A*). However, co-incubation of the same APC concentration with PF4 reduced the inhibition of thrombin generation conferred by APC in a dose-dependent

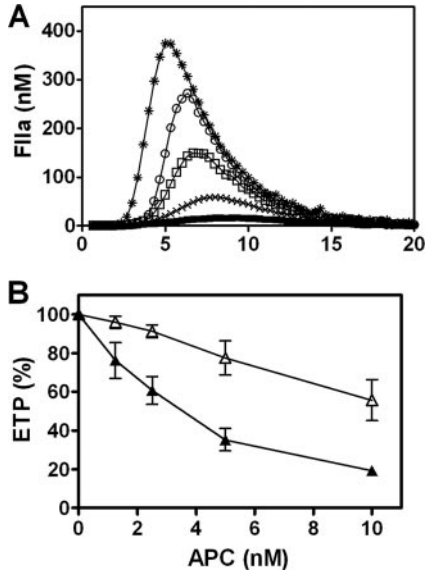

FIGURE 1. **PF4 inhibits APC anticoagulant function in platelet-poor plasma.** Coagulation was initiated in protein C-deficient plasma with 5 pM tissue factor, 4 μM phospholipid vesicles (PC/PS/PE, 60%/20%/20%), and 6.67 mM $CaCl_2$, and thrombin generation followed using a fluorogenic substrate, as described under "Experimental Procedures." *A*, thrombin generation in the absence of APC (★) was attenuated in the presence of 10 nM APC (■). Increasing PF4 concentration (0.5–2 μM) in the presence of 10 nM APC-impaired APC anticoagulant response: 0.5 μM PF4 (×), 1 μM PF4 (□), and 2 μM PF4 (○). *B*, inhibition of APC anticoagulant function in normal plasma was assessed both in the presence (△) and absence (▲) of 1 μM PF4 using respective mean ETPs ± S.E.

manner (Fig. 1*A*). In the presence of 1 μM PF4 (a concentration at which protein C activation on endothelial cells is significantly enhanced (21, 22)) the anticoagulant activity of 10 nM APC was significantly impaired, as thrombin generation was reduced to only 44 ± 4% of the original ETP, compared with 9 ± 4% in the absence of PF4 (Fig. 1*A*). To assess whether the presence of zymogen protein C altered the inhibitory activity of exogenous APC, the same experiment was performed in the presence of normal pooled plasma. Similar results were observed in APC-incubated normal pooled plasma in the presence of 1 μM PF4 (Fig. 1*B*). The same experiment was performed in the presence of a higher phospholipid concentration (25 μM), to exclude the possibility that the cationic PF4 inhibited APC anticoagulant activity by binding to and blocking access to crucial anionic phospholipid binding sites. However, PF4 was found to inhibit APC function in a similar manner (data not shown). The IC_{50} for PF4 inhibition of APC anticoagulant function was 12 μg/ml (0.4 μM), similar to PF4 serum concentration after platelet activation (22). Interestingly, PF4 in the absence of APC also mildly inhibited thrombin generation (<25% at 1 μM PF4) (data not shown). This impairment was not mediated by PF4 inhibition of contact activation, because corn trypsin inhibitor, which eliminates the contribution of contact pathway activation to thrombin generation in this system, had no effect on the observed mild reduction in thrombin generation.

Cationic Polypeptides Induce Diverse Functional Effects upon APC Anticoagulant Activity—To determine whether PF4 impairment of APC anticoagulant function represented a general functional consequence of cationic molecule association with APC, we assessed APC anticoagulant response in normal

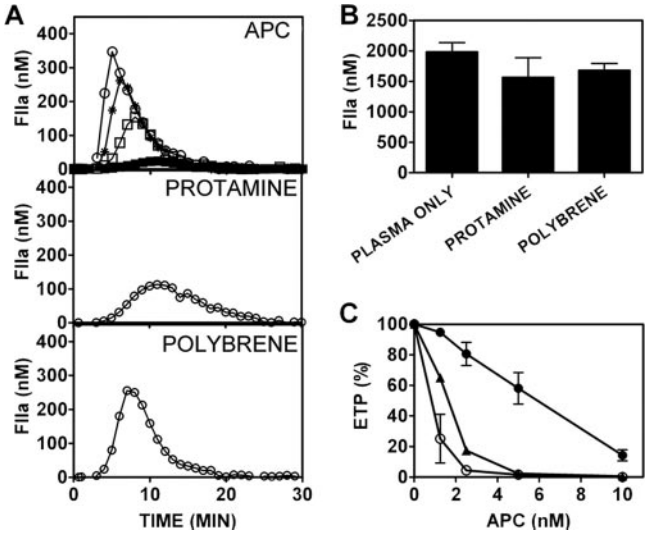

FIGURE 2. **Cationic polypeptides rapidly enhance APC anticoagulant function.** *A*, APC anticoagulant function was determined in plasma in the presence of APC (2.5–10 nM) (*top panel*: no APC (○), 2.5 nM APC (⋆), 5 nM APC (□), and 10 nM APC (■). 30 μg/ml protamine sulfate (*middle panel*) and Polybrene (*lower panel*), both (○) in the absence of APC was assessed. *B*, the ETP determined in normal plasma after thrombin generation in the presence of 30 μg/ml protamine sulfate and Polybrene was compared with that of normal pooled plasma alone. *C*, the susceptibility to APC anticoagulant activity with increasing APC concentration (1.5–10 nM APC) in the presence of protamine sulfate (▲) or Polybrene (○) was assessed and compared with thrombin generation in the presence of APC alone (●).

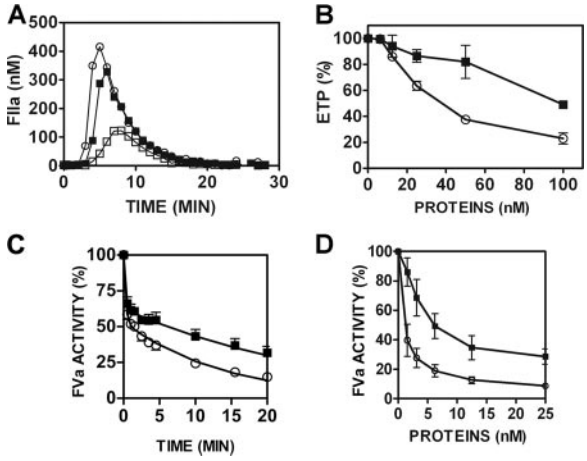

FIGURE 3. **PF4 inhibition of APC anticoagulant activity occurs via inhibition of APC-mediated FVa proteolysis.** *A*, protein S-dependent APC anticoagulant activity was measured in protein S-deficient plasma, as described under "Experimental Procedures." 5 nM APC alone (○) was compared with 5 nM APC plus 50 nM protein S in the presence (■) and absence (□) of PF4 (1 μM). *B*, protein S (6.25–100 nM)-dependent APC anticoagulant activity was assessed in the presence (■) and absence of PF4 (○) using protein S-deficient plasma. *C*, protein S-independent FVa proteolysis by APC was assessed in both the presence and absence of PF4 (1 μM). Recombinant FVa (0.8 nM final concentration) was incubated with phospholipid vesicles (PS/PE/PC, 10%/20%/70%, 25 μM final concentration) in the presence (■) or absence (○) of 1 μM PF4 in HNBSACa buffer (25 mM Hepes, 150 mM NaCl, pH 7.7, with 5 mg/ml BSA, and 5 mM $CaCl_2$. APC (1.65 nM final concentration) was added to initiate FVa inactivation, and aliquots were drawn at set time intervals over a 20-min time course. FVa inactivation was stopped by 1/25 dilution in ice-cold HNBSACa. The FVa activity was measured using a prothrombinase assay (5 nM FXa for 0.5 nM FXa, 0.5 μM prothrombin, and phospholipid vesicles (PC/PS, 10%/90%, 50 μM final concentration)). After 2 min the reaction was stopped with 40-fold dilution in ice-cold EDTA buffer (50 mM Tris-HCl, 100 mM NaCl, 20 mM EDTA, 1% polyethylene glycol 6000, pH 7.9). The thrombin formed was measured using a thrombin-specific chromogenic substrate BIOPHEN CS-01(38). The residual FVa activity was determined by comparison to the FVa activity observed prior to APC incubation. The plotted values represent the mean of at least three individual experiments ± S.E. *D*, protein S-dependent FVa proteolysis was measured in the presence (■) and absence (○) of PF4. Human protein S (1.25–25 nM) was incubated with 0.8 nM APC, 8 nM FVa, and 75 μM phospholipid vesicles (PC/PS/PE, 60%/20%/20%) in 40 mM Tris-HCl, pH 7.4, 140 mM NaCl, 3 mM $CaCl_2$, 0.3% (w/v) bovine serum albumin (0.2 nM APC, 2 nM FVa, and 19 μM phospholipid vesicles, final concentrations) for 2 min at 37 °C. A 2-μl aliquot was added to 0.3 nM FXa, 1.5 μM prothrombin, and 75 μM phospholipid vesicles (0.1 nM FXa, 0.5 μM prothrombin, and 25 μM phospholipid vesicles, final concentrations) at 37 °C for 3 min. 5 μl of ice-cold 250 mM EDTA stopped the reaction. The rate of thrombin chromogenic substrate cleavage was then measured as before.

platelet-poor plasma in the presence of two other well characterized cationic polypeptides, protamine sulfate and Polybrene. APC impaired thrombin generation in a dose-dependent fashion as previously described (25) (Fig. 2*A*, *top panel*). In the absence of APC, protamine sulfate and Polybrene both altered the dynamics of thrombin generation by reducing the peak thrombin generation and extending the time over which thrombin was generated (Fig. 2*A*, *middle* and *lower panels*). However, overall ETP was not significantly different to that determined in the absence of either cationic molecule (Fig. 2*B*).

Surprisingly, and in direct contrast to the inhibition of APC anticoagulant activity observed in the presence of PF4, both protamine sulfate and Polybrene rapidly enhanced APC anticoagulant activity. In the presence of either protamine sulfate or Polybrene (both 30 μg/ml), thrombin generation in normal plasma was entirely ablated in the presence of 5 nM APC, whereas APC alone under the same conditions only reduced thrombin generation by 41% (Fig. 2*C*). These findings demonstrate that the inhibitory effect of PF4 upon APC anticoagulant function is not solely mediated by its cationic nature.

PF4 Impairs Protein S-dependent APC Anticoagulant Activity—APC binds to anionic phospholipids, and in complex with its cofactor protein S, down-regulates thrombin generation by inactivation of procoagulant cofactors FVa and FVIIIa (6, 7). To investigate the one or more mechanisms by which PF4 inhibit APC anticoagulant activity, APC anticoagulant function was assessed in protein S-deficient plasma reconstituted with plasma-purified protein S. 10 nM APC in protein S-deficient plasma had no effect upon thrombin generation, as previously described (29) (Fig. 3*A*). However, co-incubation with protein S (6.25–100 nM) facilitated a protein S-dependent impairment of thrombin generation (IC_{50} = 36 nM, Fig. 3, *A* and *B*). Co-incubation of 1 μM PF4 with APC/protein S significantly impaired protein S-enhancement of APC anticoagulant activity in plasma ~3-fold (IC_{50} = 96 nM, Fig. 3, *A* and *B*). These data suggest that PF4 inhibition of APC anticoagulant activity in plasma is mediated in part by impairment of protein S cofactor enhancement of APC.

APC anticoagulant activity is principally determined by its ability to initiate FVa proteolysis. To gain further insight into PF4 inhibition of APC anticoagulant function, the rate of FVa proteolysis in the presence or absence of protein S and/or PF4 was determined using an APC-mediated FVa degradation reaction dependent upon the presence of anionic phospholipids for efficient APC degradation of FVa. In preliminary experiments, PF4 was shown not to influence thrombin generation by the prothrombinase reaction used in this assay (data not shown). PF4 was found to impair APC-mediated FVa proteolysis, in

particular the final stage of the characteristic biphasic FVa inactivation response (Fig. 3*C*).

A modified version of this assay was used to evaluate the ability of PF4 to inhibit FVa proteolysis by APC in the presence of protein S. PF4 inhibited APC protein S-dependent FVa proteolysis ~5-fold (Fig. 3*D*), based upon the protein S concentration required to enable half-maximal FVa inactivation in the presence and absence of PF4 (6.1 nM *versus* 1.2 nM protein S, respectively). Collectively, these results indicate that, in both plasma and purified factor assays, PF4 impairs protein S-cofactor-enhanced APC anticoagulant activity. These data are consistent with previous reports demonstrating interaction between PF4 and the Gla domain of protein C/APC and further suggest that this interaction may involve the C-terminal portion of the APC Gla domain.

PF4 Impairs Arg^{306} but Not Arg^{506} Cleavage on FVa in the Presence and Absence of Protein S—To assess the potential mechanisms by which PF4 might impair APC anticoagulant activity, the ability of APC to inactivate recombinant FVa variants in which specific APC cleavage sites (Arg^{506} and Arg^{306}) were substituted to prevent cleavage was assessed. Degradation of FVa variant FVa-R306Q/R679Q, in which only Arg^{506} cleavage by APC can occur, was unaffected by the presence of PF4, in either the presence or absence of protein S (data not shown). However, FVa degradation of FVa-R506Q/R679Q, in which only Arg^{306} cleavage takes place, was impaired by the presence of 1 μM PF4 in both the presence and absence of protein S (Fig. 4, *A* and *B*). Calculation of the rate constant for Arg^{306} cleavage by APC in FVa-R506Q/R679Q shows that the kinetic rate of Arg^{306} cleavage by APC in both the presence and absence of protein S was reduced by 3-fold (Fig. 4, *C* and *D*). PF4 therefore impairs APC anticoagulant activity in both plasma and purified protein assays via inhibition of the rate of Arg^{306} cleavage during FVa degradation by APC.

PF4 Does Not Directly Influence APC Cytoprotective Function—APC bound to EPCR via its Gla domain can activate PAR-1 on endothelial cells, triggering complex intracellular signaling that result in anti-inflammatory and anti-apoptotic cellular responses (28, 30). To ascertain whether PF4 interaction with the protein C/APC Gla domain might impair APC-EPCR-PAR-1 cytoprotective signaling, an *in vitro* assay of endothelial cell barrier permeability was used. As previously reported, thrombin markedly enhanced endothelial barrier permeability, compared with untreated cells (Fig. 5*A*). However, preincubation with APC significantly attenuated this thrombin-induced increase in permeability ($p < 0.05$, Fig. 5*A*). PF4 alone (up to 1 μM) had no independent effect upon endothelial barrier permeability, and did not protect against thrombin-mediated increased permeability. Finally, in contrast to its inhibition of APC anticoagulant activity, PF4 did not significantly inhibit the endothelial barrier protective properties of APC.

Previous studies have demonstrated that APC regulates expression of both pro- and anti-apoptotic genes to confer an anti-apoptotic phenotype in endothelial cells. Moreover, these effects are also mediated via APC-EPCR-PAR1 signaling. To determine whether PF4 might interfere with APC-mediated cytoprotection, apoptosis in EAhy926 cells was assessed by reverse transcription-PCR quantification of pro-apoptotic

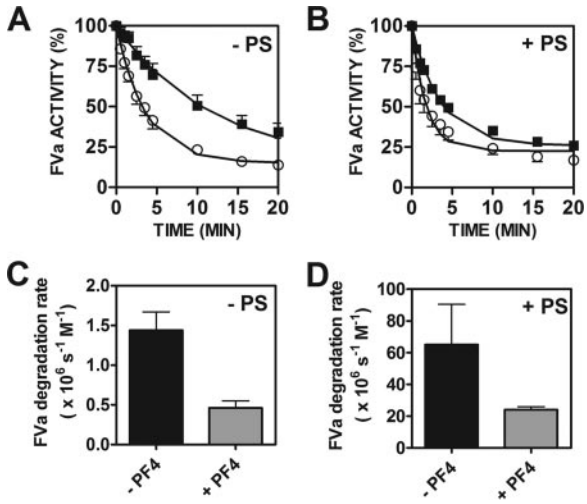

FIGURE 4. **PF4 impairs proteolysis of FVa via inhibition of APC cleavage at position Arg^{306}.** Recombinant FVa variant (506Q/679Q) (0.8 nM final concentration) was incubated with PS/PE/PC (10%/20%/70%) phospholipid vesicles (25 μM final concentration) without (*A*) or with (*B*) protein S (100 nM), in the absence (○) or presence (■) of 1 μM PF4. APC 3.3 nM (*A*) or 0.2 nM (*B*) was added to start FVa inactivation and at specified time intervals, samples were drawn, and the FVa inactivation was stopped by 1/25 dilution in ice-cold HNBSACa buffer. The FVa activity was measured with a prothrombinase assay including 5 nM FXa, 0.5 μM prothrombin, and 50 μM phospholipids (PC/PS, 90%/10%, all final concentrations) for 2 min, then stopped with EDTA buffer. The FVa activity was related to the FVa activity observed in the absence of APC. The plotted values represent the mean of at least three individual experiments ± S.E. The effect of PF4 upon rate constants for cleavage at Arg^{306} (k_{306}) using FVa degradation curves for FVa-R506Q/R679Q in the absence (*C*) or presence (*D*) of protein S was assessed. APC-mediated FVa degradation in the absence (*black bars*) or presence (*gray bars*) of PF4 (1 μM) was determined. Each *bar* represents the mean kinetic rate constant value of at least three independent experiments ± S.E.

(*Bax*) and anti-apoptotic (*Bcl-2*) gene expression following incubation with staurosporine, a well established inducer of apoptosis in endothelial cells (Fig. 5*B*). Staurosporine (20 μM) increased the *Bax/Bcl-2* ratio significantly compared with untreated cells ($p < 0.05$). Pre-treatment of EAhy926 cells with APC, however, decreased the *Bax/Bcl-2* ratio close to that determined for untreated EAhy926 cells. Furthermore, the EPCR dependence of APC-mediated cytoprotection was confirmed using an inhibitory anti-EPCR antibody (RCR-252), which entirely ablated the anti-apoptotic effect of APC. PF4 alone, or in combination with APC, had no effect upon apoptosis-related gene expression as determined by alteration of *Bax/Bcl-2* expression ratios in response to staurosporine (Fig. 5*B*). Therefore, despite PF4 enhancement of protein C activation, we demonstrate that PF4 impairs the anticoagulant response of APC by interfering with protein S cofactor enhancement. Furthermore, PF4 does not directly alter APC-mediated cytoprotection, despite its described association with the protein C/APC Gla domain.

DISCUSSION

Together with thrombospondin and β-thromboglobulin, PF4 comprises one of the major constituents of platelet α-granules (20). A number of procoagulant functions have been ascribed to PF4 that are associated with its acute release at sites of vascular injury and platelet activation. In particular, the cationic properties of PF4 facilitate high affinity heparin binding (K_D = 4 nM) and thereby attenuates the antithrombin-medi-

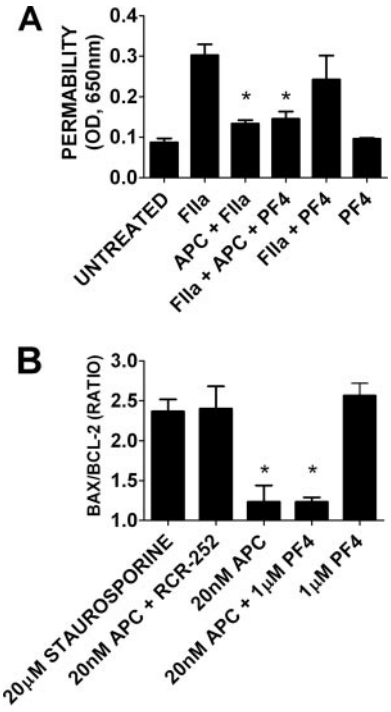

FIGURE 5. **PF4 does not alter APC-EPCR-PAR-1 cytoprotective signaling.** *A*, the effect of PF4 upon APC attenuation of thrombin-induced endothelial cell hyperpermeability was assessed as described under "Experimental Procedures." *B*, the anti-apoptotic activity of APC in the presence of PF4 was measured by determination of *Bax/Bcl-2* ratios in staurosporine-treated EAhy926 cells, as described previously (*, $p < 0.05$ when compared with staurosporine-treated EAhy926 cells).

ated anticoagulant properties of heparin and endothelial cell surface glycosaminoglycans (31, 32). Furthermore, PF4 inhibits factor XII activation by negatively charged surfaces and enhances platelet aggregation (20). In contrast, more recent studies have demonstrated that PF4 significantly enhances generation of anticoagulant APC on endothelial cell surfaces (21). Enhanced APC generation involves PF4 binding to thrombomodulin via *O*-linked chondroitin sulfate glycosaminoglycans and binding to the protein C Gla domain (23). This domain also mediates protein C binding to EPCR (33), negatively charged phospholipids, and protein S (25, 34), all of which are required for normal APC anticoagulant activity.

In vivo studies that investigated the role of PF4 upon APC generation found that low dose thrombin infusion with PF4 increased APC generation in primates 2- to 3-fold (21). However, a PF4 knockout mouse exhibited platelet aggregation defects and impaired thrombus formation, suggesting a primarily prothrombotic role for PF4 (35). Curiously, a transgenic mouse line that overexpressed PF4 exhibited similar thrombotic defects to those described for those mice lacking PF4 (35). Therefore, although PF4 appears to have a significant role in APC generation on the endothelial cell surface following platelet activation, its release from platelets is required to facilitate normal platelet aggregation and stable thrombus formation.

In this study, we demonstrate that PF4 inhibits APC anticoagulant function (Fig. 1) at PF4 concentrations described to mediate enhanced protein C activation on the surface of endothelial cells (21). Interestingly, this anticoagulant effect was specific for PF4, and was not apparent for other cationic polypeptides (*e.g.* protamine sulfate and Polybrene) (Fig. 2). Consequently, the mechanism through which PF4 impairs APC anticoagulant functions is not strictly attributable to nonspecific cationic interactions. Using purified protein assays to study APC anticoagulant activity, we observed that the inhibitory effects of PF4 were apparent when FVa inactivation by APC was assessed in the presence and absence of protein S (Fig. 3). Furthermore, despite PF4 interaction with the protein C/APC Gla domain, PF4 does not appear to interfere with the ability of the APC Gla domain to concurrently interact with negatively charged phospholipids. Instead, we demonstrate that the molecular mechanism through which PF4 inhibits APC anticoagulant function is via inhibition of the rate of Arg^{306} cleavage in FVa, which is specifically enhanced by the presence of protein S (Fig. 4).

Although protein S constitutes an essential cofactor for APC anticoagulant function in normal plasma, the protein S binding sites on APC are not well defined. However, the APC Gla domain has been shown to play a key role in mediating protein S cofactor enhancement. Recent studies have highlighted the potential importance of APC Gla residues 33–39. Although no specific PF4-Gla domain binding site has been identified, significant amino acid sequence conservation exists between the protein C/APC Gla domain and that of other vitamin K-dependent coagulation glycoproteins (36). Furthermore, previous studies demonstrated that PF4 also binds to the Gla domains of prothrombin, factor X, and protein S, although the comparative affinities of each interaction are unknown (23). It remains unclear whether the ability of PF4 to inhibit the normal protein S-APC cofactor enhancement is mediated primarily through PF4 binding to the APC Gla domain, or whether PF4 binding to the protein S Gla domain is also important.

In view of the potent anticoagulant properties of APC, the observation that PF4 increases APC generation is clearly at odds with its apparent procoagulant function. Moreover, the physiological benefit of PF4 promoting enhanced APC generation, while simultaneously inhibiting the anticoagulant function of APC, would appear somewhat counterintuitive. However, recent studies have clearly demonstrated that, in addition to its anticoagulant properties, APC bound to EPCR via its Gla domain can initiate PAR-1-mediated cytoprotective signaling on the surface of endothelial cells. *In vivo* beneficial effects of APC have been observed in a series of different animal disease models, including severe sepsis, ischemic stroke, and experimental autoimmune encephalomyelitis (13, 15, 37, 38). Also, intravenous infusion of recombinant APC significantly increased overall survival (risk reduction, 19.4%) in patients with severe sepsis in The Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis study (15). Accumulating evidence suggests that the efficacy of APC therapy in this setting primarily relates to its cytoprotective signaling activity, rather than its anticoagulant function (39). Nevertheless, the anticoagulant properties of APC are also of clinical importance, because APC therapy is associated with significant bleeding risk, restricting the dose and duration of recombinant APC administration (15). In an effort to reduce this risk of bleeding, recent studies have sought to develop APC variants that retain normal beneficial cytoprotective signaling but have reduced anticoagulant properties (40–42). In this study, we

demonstrate that, although PF4 specifically inhibits APC anticoagulant activity, APC generated in the presence of PF4 can still initiate PAR-1 cytoprotective signaling. Moreover, this observation is supported by recent elegant *in vivo* studies demonstrating that endogenously released PF4 from activated platelets stimulates APC generation, which reduces mortality following lipopolysaccharide challenge in mice (43).

Inherited or acquired resistance to APC anticoagulant activity in plasma constitutes the most common laboratory abnormality identified in patients with venous thromboembolism. In a majority of clinical cases, APC anticoagulant activity is caused by the FV Leiden polymorphism (R506Q). However, it is also well recognized that an acquired APC anticoagulant activity phenotype is observed in the presence of freeze-thawed platelets (44). Although a number of different potential mechanisms have been proposed (17, 19, 44, 45), the one or more molecular events underlying platelet-mediated APC resistance are not well defined. We demonstrate that PF4 inhibits APC anticoagulant function, and is therefore predicted to contribute to APC inhibition in the presence of activated platelets. Although normal plasma PF4 concentrations are low, significant amounts are secreted from activated platelets, so that concentrations in the vicinity of the platelet plug can reach ~10 μg/ml. Thus, our findings are in keeping with previous studies demonstrating that platelet activation is a requirement for the phenotype of platelet-mediated APC resistance (19, 44, 46).

In summary, we have shown that PF4 inhibits APC anticoagulant function, while retaining PAR-1-mediated cytoprotective signaling. This provides a rationale for how PF4 can exert prothrombotic effects, but also mediate enhanced APC generation on the surface of endothelial cells to induce both anti-inflammatory and anti-apoptotic events. Based on these observations, we propose that PF4 acts as a critical regulator of APC generation *in vivo* but also targets APC toward cytoprotective, rather than anticoagulant functions at sites of vascular injury.

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