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ORIGINAL ARTICLE

Anti–β2-glycoprotein I and anti-phosphatidylserine/prothrombin antibodies interfere with cleavage of factor V(a) by activated protein C

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Abstract

Background: The acquired thrombotic risk factor known as lupus anticoagulant (LA) interferes with laboratory clotting assays and can be caused by autoantibodies against β2-glycoprotein I (β2GPI) and prothrombin. LA is associated with activated protein C (APC) resistance, which might contribute to thrombotic risk in patients with anti-phospholipid syndrome. How antibodies against β2GPI and prothrombin cause APC resistance is currently unclear.

Objectives: To investigate how anti-β2GPI and antiphosphatidylserine/prothrombin (PS/PT) antibodies induce APC resistance.

Methods: The effects of anti-β2GPI and anti-PS/PT antibodies on APC resistance were studied in plasma (of patients with antiphospholipid syndrome) and with purified coagulation factors and antibodies.

Results: APC resistance was observed in LA-positive patients with anti-β2GPI or anti-PS/PT antibodies and in normal plasma spiked with monoclonal anti-β2GPI or anti-PS/PT antibodies with LA activity. Analysis of factor (F)V cleavage patterns after APC incubation indicated that anti-β2GPI antibodies attenuated APC-mediated FV cleavage at R506 and R306. APC-mediated cleavage at R506 is required for FV cofactor activity during inactivation of FVIIIa. Assays with purified coagulation factors confirmed that anti-β2GPI antibodies interfered with the cofactor function of FV during FVIIIa inactivation but not with FVa inactivation. Anti-PS/PT antibodies attenuated APC-mediated FVa and FVIIIa inactivation. Analysis of FV(a) cleavage patterns after APC incubation indicated that anti-PS/PT antibodies interfere with APC-mediated cleavage of FV at positions R506 and R306.

Conclusion: Anti-β2GPI antibodies with LA activity contribute to a procoagulant state by causing APC resistance via interference with the cofactor function of FV during

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FVIIIa inactivation. LA-causing anti-PS/PT antibodies interfere with the anticoagulant function of APC by preventing FV(a) cleavage.

KEYWORDS
activated protein C resistance, antiphospholipid antibodies, β2-glycoprotein I, lupus anticoagulant, prothrombin

1 | INTRODUCTION

The antiphospholipid syndrome (APS) is characterized by recurrent thrombotic events or pregnancy-related complications in patients with persistent antiphospholipid antibodies [1–3]. Thrombotic events in patients with APS can occur in venous, arterial, or microvascular circulation, with the first being the most common in the Western world [4]. This diffuse thrombotic predisposition clearly indicates a multifactorial influence of antiphospholipid antibodies in the hemostatic system [5]. Antiphospholipid antibodies are autoantibodies directed toward phospholipids or plasma proteins, which include anticardiolipin antibodies, anti–β2-glycoprotein I (β2GPI) antibodies, and antibodies that prolong the *in vitro* plasma clotting time in a phospholipid-dependent way, a phenomenon known as lupus anticoagulant (LA) [6]. Among antiphospholipid antibodies, the ones that cause LA are most strongly related to the clinical manifestations of APS [7,8]. Although laboratory assays for LA do not allow identification of the responsible antibody types, LA has been attributed to autoantibodies directed against β2GPI or prothrombin [9–11]. The clear association of LA with thrombosis represents a paradox because prolonged clotting times are usually indicative of a bleeding tendency. We have previously shown that anti-β2GPI and anti-phosphatidylserine/prothrombin (PS/PT) antibodies cause LA through different mechanisms of action. While anti-β2GPI antibodies interfere with factor (F)V activation by FXa through a direct interaction with FV, anti-PS/PT antibodies compete with FXa for phospholipid binding sites [12]. These mechanisms result in decreased thrombin formation in sensitive laboratory clotting assays, leading to the characteristic prolongation of the clotting time observed in patients with LA. How these anti-β2GPI and anti-PS/PT antibodies contribute to thrombotic risk in patients with APS is currently unclear.

One of the proposed mechanisms that explain how antiphospholipid antibodies cause thrombosis is through interference with the anticoagulant activity of activated protein C (APC), resulting in acquired APC resistance [13–22]. APC regulates coagulation through proteolytic cleavage of FV, FVa, and FVIIIa. APC-mediated cleavage of FV and FVa can occur at 3 different sites in the heavy chain of the (pro)cofactor: at Arg-306 (R306), Arg-506 (R506), and Arg-679 (R679) [23]. Cleavage of FV at R506 is essential for its cofactor activity during the APC-mediated inactivation of FVIIIa, whereas cleavage at R506 in FVa results in partial inactivation [23]. Cleavage of FVa at position R306 is required for complete FVa inactivation [23]. The importance of proteolytic cleavage of FV and FVa by APC is illustrated by the FV Leiden genetic variant, in which the R506Q amino acid substitution prevents APC-mediated cleavage of FV and FVa at position R506, resulting in an inherited form of APC resistance [24]. FV Leiden is associated with a 5- to 10-fold increased risk of venous thrombosis in heterozygous carriers and with an up to 80-fold increased risk in homozygous carriers [16,25,26]. APC resistance due to other causes is associated with an increased risk of thrombosis as well [13,27]. The underlying mechanism through which antiphospholipid antibodies cause APC resistance remains unknown. Here, we aimed to investigate how LA and specifically anti-β2GPI and anti-PS/PT antibodies interfere with the anticoagulant response to APC by performing in-depth molecular analysis.

Essentials

- Lupus anticoagulant is associated with activated protein C (APC) resistance.
- Anti–β2-glycoprotein I and antiphosphatidylserine/prothrombin antibodies cause APC resistance.
- Anti–β2-glycoprotein I antibodies interfere with the cofactor function of factor (F)V in FVIIIa inactivation by APC.
- Antiphosphatidylserine/prothrombin antibodies interfere with APC-mediated FV(a) cleavage.

2 | MATERIALS AND METHODS

2.1 | Materials

Pooled normal plasma (PNP) was prepared from at least 250 healthy hospital workers from the University Medical Center Utrecht, the Netherlands. Institutional ethics review board approval was obtained, and all subjects gave written informed consent. Monoclonal anti-β2GPI antibodies 3B7 and EM6 and anti-PS/PT antibodies 3B1 and 11H2 were produced in-house and purified from hybridoma culture medium with protein G Sepharose (GE Healthcare). Prothrombin active site mutant (FIIi S525A) was produced in human embryonic kidney 293 cells in the presence of vitamin K1 and purified from serum-free medium with a calcium-dependent monoclonal antibody against prothrombin. Dilute Russell's viper venom time (dRVVT)

screen and confirm reagents were obtained from Stago. HemosIL silica clotting time (SCT) screen and confirm reagents as well as chromogenic substrates for FXa (S2222) and thrombin (S2238) were obtained from Werfen/Instrumentation Laboratories. Protac and Pefabloc TH were obtained from Pentapharm. Phospholipids (coagulation reagent I) were obtained from Avanti Polar Lipids and prepared as described before [9]. Human FV, FVa, FX, FXa, APC, protein S, and monoclonal anti-FV antibody 5146 against FV heavy chain were obtained from Haematologic Technologies. Recombinant human FVIII (Advate; Baxalta) was obtained as pharmaceutical formulation. Hirudin was obtained from Merck. Human prothrombin, α-thrombin, and FIXa were obtained from Enzyme Research Laboratories. Human β2GPI was purified from fresh citrated plasma as described previously [11]; purity was checked with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. All β2GPI preparations yielded a single band of 50 kDa and were >99% pure. IRDye 800CW donkey anti-mouse secondary antibody was obtained from LI-COR Biosciences. QUANTA Lite anti-PS/PT immunoglobulin G (IgG)/immunoglobulin M (IgM) was obtained from Inova Diagnostics. IBL Cardiolipin IgG/IgM enzyme-linked immunosorbent assay (ELISA) kit was obtained from IBL International GmbH. IMTEC β2GPI antibodies IgG/IgM ELISA kit was obtained from Clindia Benelux BV.

2.2 | Plasma samples

Leftover citrated plasma samples from patients with thrombotic APS on vitamin K antagonists were used. Plasma was collected as part of a single-center cross-sectional observational study that has been approved by the local medical ethical committee of University Medical Center Utrecht [28]. Written informed consent was obtained from all patients. Plasma samples had been tested for LA using dRVTT and activated partial thromboplastin time. Samples were mixed with PNP to correct for coagulation factor deficiencies. Normalized LA (nLA) ratios were expressed as follows: (screen clotting time of patient/screen clotting time of PNP)/(confirm clotting time of patient/confirm clotting time of PNP). Anti-β2GPI antibodies were determined with the IMTEC β2GPI antibodies IgG/IgM ELISA kit and anticardiolipin antibodies with the IBL Cardiolipin IgG/IgM ELISA kit. Samples were deemed positive for anti-β2GPI antibodies when they were positive for anti-β2GPI antibodies, anticardiolipin antibodies, or both. Anti-PS/PT IgG and IgM were measured with the QUANTA Lite kit according to the instructions of the manufacturer.

2.3 | Coagulation assays

All coagulation assays were performed on an MC10-plus coagulometer (Merlin Medical) at 37 °C. LA activity was measured with dRVVT and SCT screen and confirm reagents. Plasma from healthy donors or patients was mixed 1:1 with PNP, or PNP was incubated with 100 μg/mL of monoclonal anti-β2GPI antibodies (3B7 and EM6) or monoclonal anti-PS/PT antibodies (3B1 and 11H2) in absence or presence of 0.25 U/mL of protein C activator (protac). Samples were incubated for 2 minutes at 37 °C prior to the addition of dRVVT screen and confirm reagents. Normalized APC sensitivity ratios (nAPCsrs) were calculated as follows: ([clotting time with protac]/[clotting time without protac] in presence of the antibody)/([clotting time with protac]/[clotting time without protac] in absence of the antibody).

2.4 | Factor Va inactivation by APC

Factor Va (0.5 nM) was incubated with 4 μM phospholipids and 0.5 nM APC in coagulation buffer (10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 140 mM NaCl, 5 mM $CaCl_2$, and 0.1% bovine serum albumin (BSA); pH 7.5) with 50 μg/mL β2GPI or prothrombin active site mutant, with or without 50 μg/mL anti-β2GPI antibody 3B7 or anti-PS/PT antibody 3B1 for 10 minutes at 37 °C. Subsequently, 50 μL aliquots were transferred into 200 μL of coagulation buffer with 0.5 nM FXa, 0.5 μM prothrombin, 10 μM phospholipids, and 1 μM Pefabloc TH and incubated for 3 minutes at 37 °C. Prothrombinase reactions were terminated by transferring 50-μL aliquots to 150 μL of 10 mM HEPES, 175 mM NaCl, 20 mM EDTA, and 0.1% BSA (pH 7.7). Thrombin formation was measured by addition of 100 μL of this mixture to 100 μL of 1 mM chromogenic substrate S2238. Thrombin concentrations were deduced from a thrombin calibration curve with known concentrations. Factor Va concentration was calculated assuming a turnover number for prothrombinase of 6000 mole thrombin per minute per mole FXa-FVa [29].

2.5 | Factor VIIIa inactivation by APC

Factor VIIIa degradation assay was performed as described [30], with a few modifications. Factor VIII (0.6 U/mL) was activated with thrombin (0.002 U/mL) in the presence of 56 μM phospholipids in 50 mM Tris, 10 mM $CaCl_2$, and 0.02% BSA (pH 7.3) at 37 °C for 2 minutes. Factor VIII activation was stopped with hirudin (20 U/mL). For FVIIIa inactivation in presence of antibody-β2GPI complexes, FVIIIa was incubated with 4 nM APC and 4 nM protein S with or without 2 nM FV, 50 μg/mL β2GPI, 50 μg/mL anti-β2GPI antibody 3B7, or combinations thereof at 25 °C. At the indicated time points, FX (10 μg/mL) was added to the reaction and incubated for an additional minute, after which reactions were terminated with EDTA (16 mM). For FVIIIa inactivation in presence of antibody-PS/PT complexes, FVIIIa was incubated with 1 nM APC, 4 nM protein S, and 50 μg/mL prothrombin active site mutant with or without 0.6 nM FV and 50 μg/mL of anti-PS/PT antibody 3B1 at 37 °C. At the indicated time points, FX (9 μg/mL) and FIXa (2.3 nM) were added to the reaction and incubated for 2 minutes at 37 °C, after which reactions were terminated with EDTA. Factor Xa formation was measured with chromogenic substrate S2222. Factor Xa concentrations were deduced from a calibration curve of FXa with known concentrations [31].

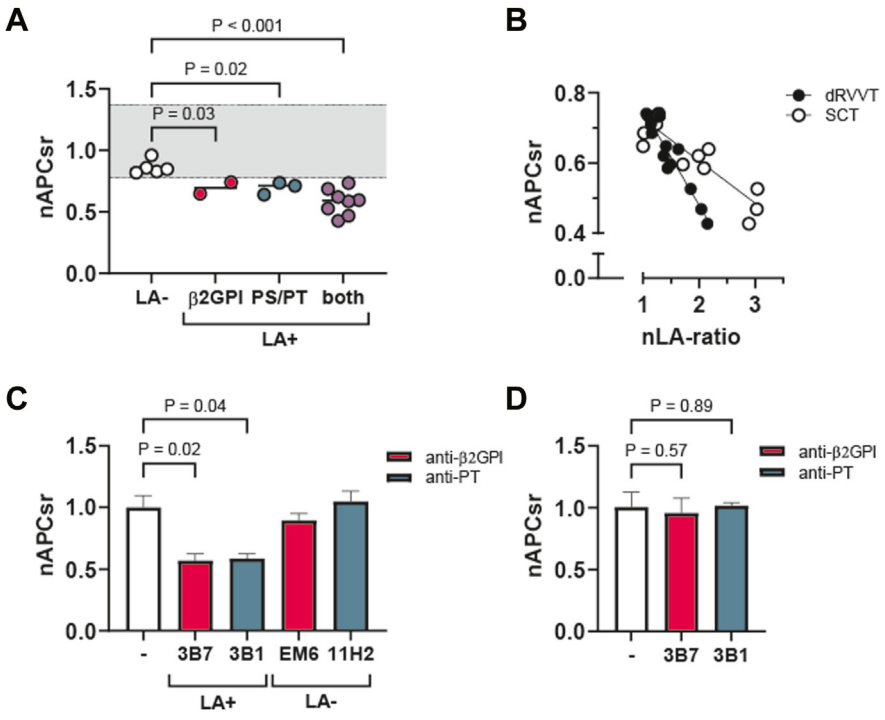

FIGURE 1 Activated protein C sensitivity in plasma with anti-β2GPI and anti-PS/PT antibodies. (A) Pooled normal plasma (PNP) was mixed 1:1 with plasma of LA-negative patients with thrombotic antiphospholipid syndrome (APS) and anti-β2GPI antibodies or anti-PS/PT antibodies (N = 5) and LA-positive patients with thrombotic APS (N = 13), of whom 2 had anti-β2GPI antibodies, 3 had anti-PS/PT antibodies, and 8 had both. Mixed plasma was supplemented with protein C activator (protac), and clotting was initiated with dRVVT screen reagents. nAPCsrs were calculated. Differences in nAPCsr between LA-negative patient plasma and LA-positive patient plasma were analyzed with unpaired Student's t-test. Shaded area indicates minimum and maximum of nAPCsr determined in plasma from healthy controls mixed 1:1 with PNP (n = 20). (B) Normalized LA ratio as a function of nAPCsr in 13 patients with thrombotic APS and LA. LA was determined with both dRVVT and SCT reagents. (C) nAPCsrs were determined in PNP spiked with monoclonal anti-β2GPI antibody (3B7) or anti-PS/PT antibody (3B1) with LA activity, or with anti-β2GPI antibody (EM6) or anti-PS/PT antibody (11H2) without LA activity. Clotting times were obtained using dRVVT screen reagents. Differences between nAPCsr with and without monoclonal antibodies were analyzed with an unpaired Student's t-test (n = 3). (D) nAPCsrs were determined in PNP spiked with monoclonal anti-β2GPI antibody (3B7) or anti-PS/PT antibody (3B1) with LA activity. Clotting times were obtained with dRVVT confirm reagents. Differences between nAPCsr with and without monoclonal antibodies were analyzed with an unpaired Student's t-test (n = 3). β2GPI, β2-glycoprotein I; dRVVT, dilute Russell's viper venom time; LA, lupus anticoagulant; nAPCsr, normalized activated protein C sensitivity ratio; nLA, normalized lupus anticoagulant; PS, phosphatidylserine; PT, prothrombin; SCT, silica clotting time.

2.6 | Western blot of factor V and factor Va inactivation by APC

Visualization of FV(a) inactivation by APC with Western blot was performed as described [23,32], with few modifications. For FV inactivation, 11 nM FV was incubated with 4 μM phospholipids in coagulation buffer with or without 50 μg/mL of β2GPI or prothrombin active site mutant and 100 μg/mL of anti-β2GPI antibody 3B7 or anti-PS/PT antibody 3B1 in the presence of 4.4 nM protein S at 37 °C. Next, 5 nM APC was added to start FV inactivation, and aliquots were taken at several time points and subjected to SDS-PAGE. For FVa inactivation, 10 nM FVa was incubated with 25 μM phospholipids, 0.7 μM prothrombin active site mutant, and 100 nM protein S in coagulation buffer with or without 100 μg/mL of anti-PS/PT antibody 3B1 at 37 °C. Next, 0.1 nM APC was added to start FVa inactivation, and aliquots were taken at the indicated time points and subjected to SDS-PAGE. After electrophoresis, the proteins were transferred onto Immobilon-FL polyvinylidene fluoride membranes (Merck Millipore).

The FV(a) bands were visualized with a mouse monoclonal antibody 5146 against FV heavy chain as a primary reagent and a donkey anti-mouse IRDye 800CW antibody as a secondary reagent. Bands were visualized on an Odyssey infrared imager (LI-COR Biosciences). Band intensity was quantified with ImageJ software (version 1.47) (U.S. National Institutes of Health).

2.7 | Statistical analysis

Results are reported as mean and SD, and all data analyses were performed with GraphPad Prism 8.3 software. Correlation between LA and APC sensitivity was analyzed with Spearman's rho (ρ). Differences between groups or treatments were analyzed with unpaired Student's *t*-tests. Effects of antiphospholipid antibodies or FV on the inactivation of FVa (antiphospholipid antibodies only) or FVIIIa (antiphospholipid antibodies and FV) by APC were analyzed with 2-way analysis of variance. *P* values of <.05 were considered statistically significant.

3 | RESULTS

3.1 | Anti-β2GPI antibodies and anti-PS/PT antibodies with LA activity cause APC resistance

APC resistance is frequently observed in patients with APS and LA and is thought to contribute to thrombotic risk [33]. LA can be caused by both anti-β2GPI antibodies and anti-PS/PT antibodies, but the contribution of these antibodies to APC resistance is unclear. To investigate the association between antibody types and APC resistance, the sensitivity for APC was assessed in 13 patients with thrombotic APS and LA, including 2 with anti-β2GPI antibodies, 3 with anti-PS/PT antibodies, and 8 with both antibody types (Figure 1A). In addition, sensitivity for APC was measured in 5 patients with thrombotic APS without LA who had anti-β2GPI or anti-PS/PT antibodies. All patients with LA had APC resistance, suggesting that both anti-β2GPI and anti-PS/PT antibodies can induce APC resistance. nAPCsrs strongly correlated with LA strength (nLA ratio) (for LA determined with dRVVT, Spearman's ρ, −0.93; 95% CI, −0.98 to −0.78; for LA determined with SCT, Spearman's ρ, −0.75; 95% CI, −0.92 to −0.33) (Figure 1B). We also evaluated the correlation between antiphospholipid antibody titers and nAPCsr. We observed a good correlation between anti-PS/PT IgG antibodies and nAPCsr (Spearman's ρ, −0.75; 95% CI, −0.92 to −0.33) and anticardiolipin IgG and nAPCsr (Spearman's ρ, −0.63; 95% CI, −0.88 to −0.11). No correlation was observed between any of the other antiphospholipid antibodies and nAPCsr: for anti-β2GPI IgG and nAPCsr, ρ, 0.03; 95% CI −0.54 to 0.58; for anti-β2GPI IgM and nAPCsr, ρ, 0.38; 95% CI, −0.23 to 0.78; for anticardiolipin IgM and nAPCsr, ρ, −0.05; 95% CI, −0.60 to 0.53; and for anti-PS/PT IgM and nAPCsr, ρ, −0.5; 95% CI, −0.83 to 0.09.

To confirm that both anti-β2GPI and anti-PS/PT antibodies cause APC resistance, APC sensitivity was assessed with dRVVT screen reagents in normal plasma spiked with monoclonal antibodies against β2GPI or prothrombin by activating protein C with protac (Figure 1C). The LA activity of these antibodies is shown in the Supplementary Table. Protac caused a 1.7-fold (SD, 0.2) prolongation of the dRVVT screen clotting time, which is in line with APC-mediated FV inactivation. Addition of an anti-β2GPI antibody with LA activity (3B7) to PNP resulted in a mean (SD) nAPCsr of 0.57 (0.06), indicating APC resistance; however, addition of an antibody without LA activity (EM6) did not (mean [SD] nAPCsr, 0.90 [0.06]). Similarly, addition of an anti-PS/PT antibody with LA activity (3B1) caused APC resistance (mean [SD] nAPCsr, 0.59 [0.04]), whereas addition of an anti-PS/PT antibody without LA activity (11H2) did not (mean [SD] nAPCsr, 1.05 [0.08]). Addition of protac to PNP caused a 1.5-fold (SD, 0.2) prolongation of dRVVT confirm clotting times, indicating normal FV inactivation at high phospholipid concentrations. APC resistance induced by antibodies with LA activity was lost when coagulation was initiated with dRVVT confirm reagents (Figure 1D). These data further support the association between LA activity of anti-β2GPI or anti-PS/PT antibodies and APC resistance.

3.2 | Anti-β2GPI antibodies cause APC resistance via inhibition of the cofactor function of FV during FVIII inactivation

Considering the interaction between β2GPI and FV or FVa [12], we investigated whether anti-β2GPI antibodies cause APC resistance through inhibition of FVa inactivation in a purified system. Hereto, APC was added to FVa, and residual FVa activity was assessed over time. Under these conditions, APC readily inactivated FVa with a mean (SD) residual FVa activity of 38% (13%) after 10 minutes (Figure 2A). Addition of β2GPI and 3B7 did not influence FVa inactivation (*P* = .588), suggesting that anti–β2GPI-induced APC resistance is not due to attenuated FVa inactivation. Since APC also degrades FVIIIa, for which FV is a cofactor, we next investigated the effects of anti-β2GPI antibodies on the inactivation of FVIIIa. APC and protein S with or without FV were added to FVIIIa, and residual FVIIIa activity was assessed over time. APC inactivated FVIIIa, with a mean (SD) residual FVIIIa activity of 83% (10%) after 1 minute and of 32% (9%) after 5 minutes. Factor V enhanced the inactivation of FVIIIa by APC (*P* = .002), with a mean (SD) residual FVIIIa activity of 42% (21%) after 1 minute and 29% (2%) after 5 minutes (Figure 2B). Addition of β2GPI and 3B7 had no effect on FVIIIa inactivation by APC (*P* = .867), but FV no longer enhanced FVIIIa inactivation (*P* = .704). These data suggest that antibody-β2GPI complexes interfere with FVIIIa inactivation by attenuating the cofactor effect of FV on APC.

FV can only function as a cofactor for APC during FVIIIa inactivation when it is cleaved by APC at position R506. To further investigate the molecular mechanism behind the attenuation of FV cofactor activity on APC-mediated FVIIIa inactivation, we analyzed the proteolytic fragments of FV generated by APC with Western blotting using a monoclonal antibody that recognizes an epitope between R306 and R506 (Figure 2C–E). In the absence of the antibody-β2GPI complex, a 75-kDa immunoreactive band appeared, corresponding to a fragment

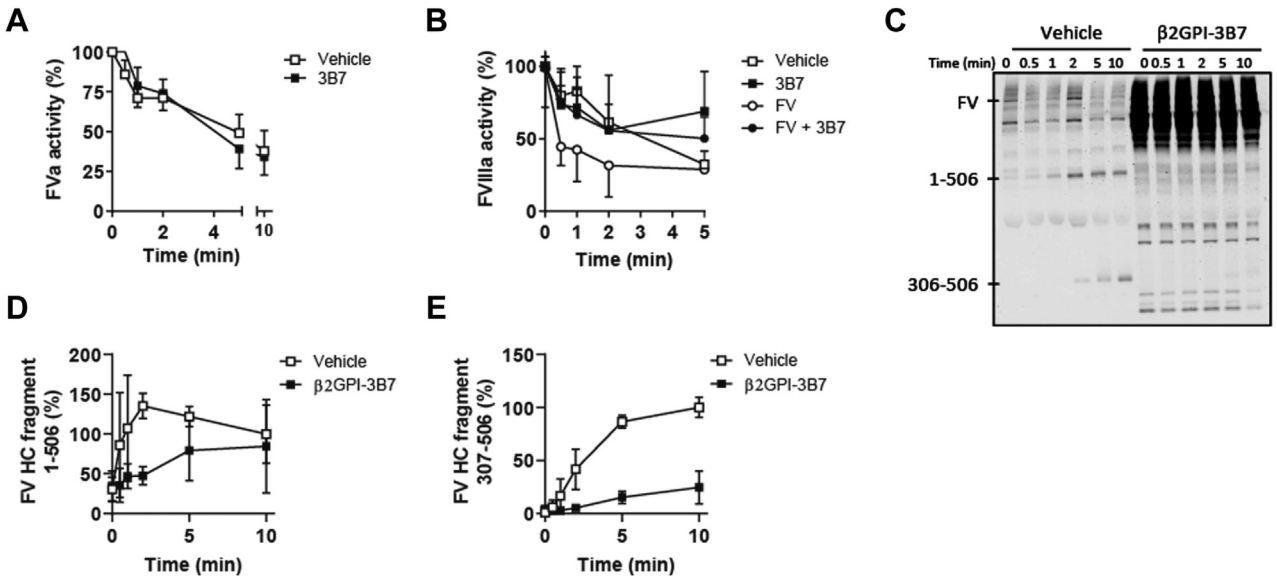

FIGURE 2 Antibody-β2GPI complexes interfere with the cofactor function of FV in activated protein C (APC)–mediated FVIIIa inactivation. (A) FVa was incubated with APC and β2GPI with or without monoclonal anti-β2GPI antibody 3B7. At the indicated time points, FVa activity was assessed. (B) FVIII was activated and was subsequently incubated with APC and protein S with (FV) or without (vehicle) FV. Alternatively, FVIIIa was incubated with APC and protein S in the presence of β2GPI and 3B7 with (FV + 3B7) or without FV (3B7). Data in (A) and (B) are expressed as mean (SD) residual FVa-activity (A) or FVIIIa-activity (B), with activity at t = 0 at 100%. Differences between conditions were analyzed with 2-way analysis of variance (n = 3). (C) Factor V was incubated with or without β2GPI and 3B7 in the presence of protein S. APC was added to start FV inactivation, and at the indicated time points, samples were drawn and analyzed by Western blotting using a monoclonal antibody AHV5146 that recognizes an epitope between Arg-306 and Arg-506 fragment as the primary antibody and an IRDye 800CW donkey anti-mouse as the secondary antibody. The secondary antibody also reacts with our monoclonal antibody, which can be seen in the samples containing β2GPI-3B7. Factor V heavy chain (HC) fragments 1-506 (75 kDa) (D) and 307-506 (30 kDa) (E) were quantified with ImageJ software. Factor V HC fragments of 1-506 or 307-506 in the presence of APC without β2GPI and 3B7 at 10 minutes were set to 100%. Differences between conditions were analyzed with 2-way analysis of variance (n = 3). β2GPI, β2-glycoprotein I; FV, factor V; FVa, factor Va; FVIIIa; factor VIIIa; HC, heavy chain.

spanning residues 1 to 506 and consistent with APC-mediated cleavage of FV at position R506. After 2 minutes, the intensity of the 1 to 506 fragment declined, and a 30-kDa band appeared, corresponding with a fragment spanning residues 307 to 506, indicating subsequent cleavage at R306. In the presence of the antibody-β2GPI complex, APC-mediated cleavage of FV at position R506 was slower (P = .002): levels of FV cleavage in absence of antibody-β2GPI complexes after 30 seconds incubation with APC were similar to those reached after 10-minute incubation with APC in presence of antibody-β2GPI complexes. Combined, these data show that antibody-β2GPI complexes interfere with proteolytic cleavage of FV by APC, resulting in impaired cofactor activity during FVIIIa inactivation.

3.3 | Anti-PS/PT antibodies cause APC resistance via enhanced protection of FVa within the prothrombinase complex

It is known that FVa inactivation by APC is inhibited by prothrombin [32,34]. To determine how anti-PS/PT antibodies cause APC resistance, we evaluated the effect of anti-PS/PT antibodies on FVa and FVIIIa inactivation by APC in a purified system. In presence of prothrombin, mean (SD) residual FVa activity was 56% (8%) after 10-minute incubation with APC (Figure 3A). Addition of anti-PS/PT antibodies inhibited FVa inactivation by APC (P = .027), with a mean (SD) residual FVa activity of 85% (3%) after 10 minutes. The effect of anti-PS/PT antibodies on FVIIIa inactivation by APC was also studied. In presence of prothrombin, mean (SD) residual FVIIIa activity was 81% (19%) after 1 minute. Factor V enhanced the inactivation of FVIIIa by APC (P = .033) (Figure 3B), with mean (SD) residual FVIIIa activity of 65% (25%) after 1 minute. Factor VIIIa inactivation was not affected by anti-PS/PT antibodies (P = .712). However, the effect of FV on FVIIIa inactivation was lost, as FVIIIa inactivation was similar in the presence or absence of FV (P = .327).

Analysis of proteolytic fragments of FVa with Western blotting showed that addition of anti-PS/PT antibodies delayed the appearance of the 307-506 fragment (P = .003) (Figure 3C, E). Cleavage of FV at R506 by APC occurred already at t = 0, as shown by the 1-506 fragment. The intensity of this fragment did not change over time. As activity assays indicated that anti-PS/PT antibodies impaired the cofactor function of FV during APC-mediated FVIIIa inactivation, we also studied the cleavage of FV by APC in presence of anti-PS/PT antibodies. The appearance of the 1-506 fragment was delayed in the presence of anti-PS/PT antibodies (P < .001) (Figure 3D, F). Subsequent cleavage of FV at position R306 did not occur at all after 10-minute incubation with anti-PS/PT antibodies (Figure 3G).

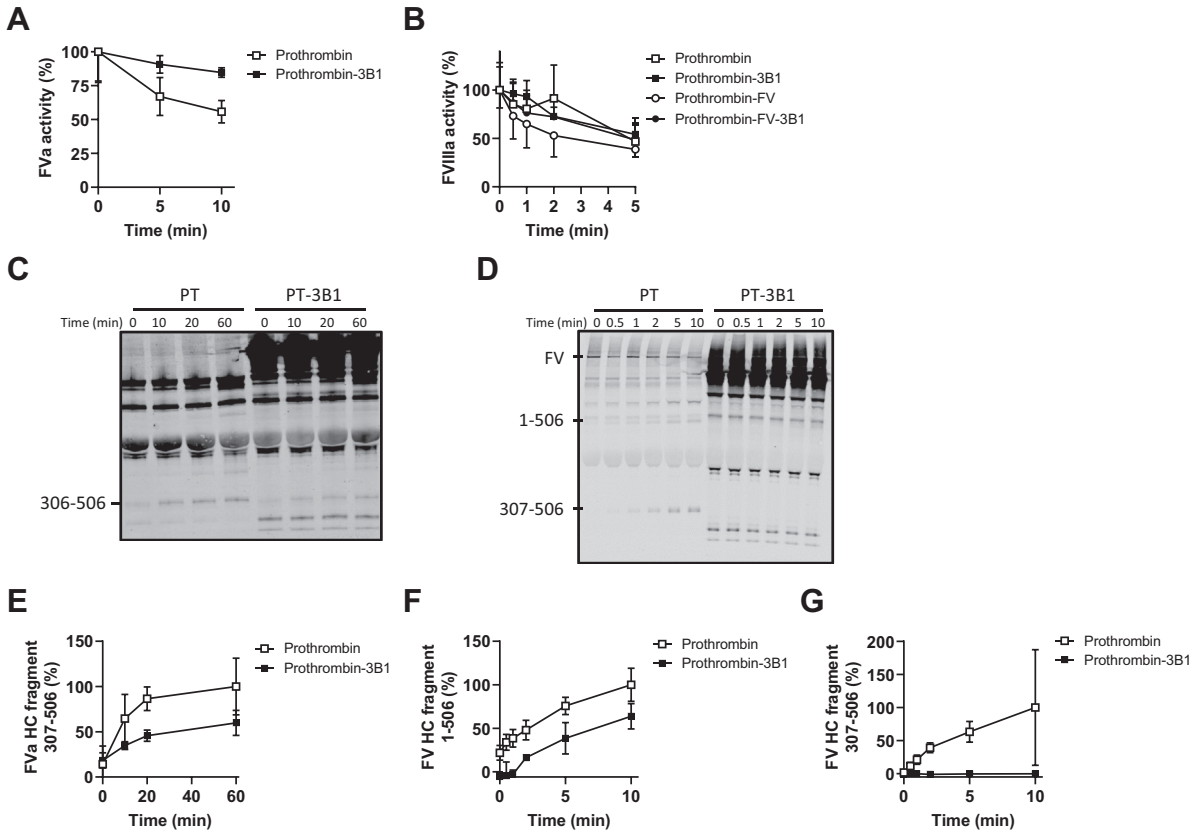

FIGURE 3 Antibody-PS/PT complexes interfere with activated protein C (APC)–mediated FV(a) cleavage. (A) FVa was incubated with APC and prothrombin with or without monoclonal anti-PS/PT antibody 3B1. At the indicated time points, FVa activity was assessed. (B) FVIII was activated and was subsequently incubated with APC, protein S, and prothrombin and with or without FV or 3B1. Data in (A) and (B) are expressed as mean (SD) residual FVa activity (A) or FVIIIa activity (B), with activity at t = 0 at 100% (n = 3). Factor Va (C) and FV (D) were incubated with or without 3B1 in the presence of prothrombin and protein S. APC was added to start FV(a) inactivation, and at the indicated time points, samples were drawn and analyzed by Western blotting. The secondary antibody also reacts with our monoclonal antibody, explaining the high molecular weight bands in the samples containing prothrombin and 3B1. Factor Va HC fragments 307-506 (75 kDa) (E) and FV HC fragments 1-506 (75 kDa) (F) and 307-506 (30 kDa) (G) were quantified with ImageJ software. Factor V(a) HC fragments of 1-506 or 307-506 in the presence of APC without 3B1 at 10 minutes were set to 100%. Differences between conditions were analyzed with 2-way analysis of variance (n = 3). FV, factor V; FVa, factor Va; FVIIIa; factor VIIIa; HC, heavy chain. PT, prothrombin.

4 | DISCUSSION

A decreased anticoagulant response to APC is defined as APC resistance, which predisposes to events of thrombosis [35]. Thromboembolic events are common manifestations in the APS population [16], yet the underlying mechanism through which antiphospholipid antibodies cause thrombosis remains unknown. Here, we showed that both anti-β2GPI antibodies and anti-PS/PT antibodies cause APC resistance, but they do so in different ways. Anti-β2GPI antibodies cause APC resistance via inhibition of APC-mediated cleavage of FV at R506. In this way, they impair the cofactor function of FV in APC-mediated degradation of FVIIIa. Anti-PS/PT antibodies interfere with cleavage at positions R306 and R506 in both FVa and FV by APC, resulting in inhibition of APC-mediated FVa and FVIIIa inactivation.

The association of the presence of antiphospholipid antibodies with APC resistance has been frequently observed in patients with thrombotic APS [14–21,36]. We found APC resistance in patients with thrombotic APS along with either anti-β2GPI antibodies, anti-PS/PT antibodies, or both. Recently, a study investigated the contribution of anti-β2GPI and anti-PS/PT antibodies to APC resistance [17]. They found an effect of affinity-purified anti-β2GPI and anti-PS/PT antibodies on APC resistance [37]. In our study, we only found an effect of antiphospholipid antibodies on APC resistance when using a limited phospholipid concentration. Similar observations were reported by a study that showed that APC resistance caused by anti-PS/PT antibodies disappeared at high phospholipid concentrations [38]. We have previously shown that FV deficiency results in less pronounced effects on dRVVT confirm clotting times than on dRVVT screen clotting times [39], indicating that protac treatment of normal plasma results in similar residual FV activity with both reagents. These data suggest that APC-mediated FV inactivation is not influenced by phospholipid concentration. The disappearance of the effect of antiphospholipid antibodies on APC resistance at a high phospholipid concentration is therefore likely related to the LA phenomenon. This is further supported by the strong correlation between the nLA ratio and the extent of APC resistance.

Several mechanisms have been proposed to explain acquired APC resistance in APS, including a direct interaction between APC and β2GPI [15]. Another possible scenario to explain APC resistance is that antiphospholipid antibodies cause APC resistance through competition with APC for binding sites on phospholipids. The affinity of APC for phospholipids is relatively low compared with that of other proteins with GLA domains such as prothrombin [40], and while the affinity of β2GPI for phospholipids is similar to that of APC, antibody-mediated avidity likely increases binding [41]. Although we did not investigate the direct interaction between APC and β2GPI, our data indicate that cleavage of FVa by APC is not influenced by the presence of antibody-β2GPI complexes, raising doubt on the relevance of a direct interaction with APC. Moreover, these data do not support competition for binding sites on phospholipids as a likely cause of APC resistance. Instead, we found that anti-β2GPI antibodies impaired the cofactor function of FV during APC-mediated FVIIIa inactivation, which fits with the direct interaction between FV and β2GPI we described previously [12]. The clinical importance of the role of FV as an APC cofactor is illustrated by the prothrombotic state associated with the R506Q mutation in FV, which can no longer function as an APC cofactor during FVIIIa degradation [23].

We also investigated the mechanism of anti-PS/PT antibodies on APC resistance. Tran et al. [32] showed that prothrombin inhibits FVa inactivation by APC-mediated cleavages at R306 and R506. We hypothesized that anti-PS/PT antibodies enhance the protection of FVa cleavage by prothrombin within the prothrombinase complex. Indeed, our results show that APC-mediated FVa inactivation is strongly inhibited in presence of both prothrombin and an anti-PS/PT antibody. Similar results were seen in a study by Pontara et al. [38], who found impaired inactivation of FVa by APC in presence of anti-PS/PT antibodies. Analysis of proteolytic fragments of FVa showed that the addition of anti-PS/PT antibodies delayed the appearance of the 307-506 fragment. As the intensity of the 1-506 fragment did not change during incubation with APC, we are not sure that both the R306 and R506 cleavages are inhibited by anti-PS/PT antibodies. However, as anti-PS/PT antibodies interfered with APC-mediated FV cleavage at both R306 and R506, we can assume that this is similar to cleavage of FVa. We previously showed that antibody-PS/PT complexes interfere with FXa for phospholipid binding sites, indicating competition for binding to phospholipids between coagulation factors and antibody-PS/PT complexes, which explains the LA phenomenon associated with these antibodies [12]. Data presented in the current study also fit with competition for phospholipid binding sites.

Thrombotic events in patients with APS can occur in every blood vessel. Moreover, risk factors associated with thrombotic complications in arterial thrombosis differ from those in venous thrombosis [42]. Although resistance to APC is a well-known risk factor for venous thrombosis, as seen in patients with FV Leiden [13,26,27], it does not seem to play an evident role in arterial thrombosis [43]. This suggests that the interference of antiphospholipid antibodies with hemostasis differs within each vessel and that multiple pathways are involved. Binding of anti-β2GPI antibodies to β2GPI causes activation of numerous cell types, including platelets, endothelial cells, and monocytes [44,45]. Therefore, we assume that the mechanism of anti-β2GPI and anti-PS/PT antibodies causing thrombosis is not limited to interference within the anticoagulant protein C pathway.

The results presented here were obtained with model monoclonal antibodies against β2GPI or prothrombin, of which the majority of the experiments were performed with only 1 monoclonal antibody against β2GPI (3B7) or prothrombin (3B1). Therefore, it remains to be determined to what extent our results apply to other monoclonal antibodies directed against β2GPI or prothrombin and whether the polyclonal heterogeneous antibody population found in patients with thrombotic APS behaves in a similar manner. We did not use human purified antiphospholipid antibodies in our experiments. It should be noted that not all patient-derived affinity-purified anti-β2GPI antibody preparations have LA activity [37,46,47]. This discrepancy might be caused by the difference in purification method, or the domain-specificity of the purified antibody preparations. In support of the applicability of our data to patients with APS and a β2GPI-dependent LA, the monoclonal antibody 3B7 binds to the first domain of β2GPI, similar to pathogenic antiphospholipid antibodies [48–50], and 3B7:β2GPI complexes strongly interact with immobilized cardiolipin. Moreover, the coagulation profile obtained with the monoclonal anti-β2GPI or anti-PS/PT antibodies was similar to the profile in 13 patients with thrombotic APS and LA who were also positive for anti-β2GPI antibodies, anti-PS/PT antibodies, or both. A substantial proportion of patients with LA do not have antibodies against β2GPI or prothrombin [37,51,52]. The mechanism responsible for the prothrombotic phenotype in patients with other subtypes of antibodies remains to be determined.

In conclusion, anti-β2GPI and anti-PS/PT antibodies with LA activity cause APC resistance, which might contribute to thrombotic risk in LA carriers. The mechanism of action of these antibodies differs: anti-β2GPI antibodies attenuate APC-mediated cleavage of FV at position R506, which results in the inability of FV to act as a cofactor in the degradation of FVIIIa by APC, whereas anti-PS/PT antibodies cause APC resistance via interfering with both FV and FVa cleavage by APC at positions R306 and R506, resulting in attenuated inactivation of FVa and FVIIIa.

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AUTHOR CONTRIBUTIONS

T.N., S.C., J.J.J., and V.v.d.V. performed the experiments and analyzed the data. T.N., R.E.G.S., M.L., P.G.d.G., J.C.M.M., and R.T.U. designed the study, interpreted data, and wrote the manuscript. All authors read and approved the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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