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Research Article **Hematology**

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Antiphospholipid antibodies promote leukocyte–endothelial cell adhesion and thrombosis in mice by antagonizing eNOS via β2GPI and apoER2

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In antiphospholipid syndrome (APS), antiphospholipid antibodies (aPL) binding to β2 glycoprotein I (β2GPI) induce endothelial cell–leukocyte adhesion and thrombus formation via unknown mechanisms. Here we show that in mice both of these processes are caused by the inhibition of eNOS. In studies of cultured human, bovine, and mouse endothelial cells, the promotion of monocyte adhesion by aPL entailed decreased bioavailable NO, and aPL fully antagonized eNOS activation by diverse agonists. Similarly, NO-dependent, acetylcholine-induced increases in carotid vascular conductance were impaired in aPL-treated mice. The inhibition of eNOS was caused by antibody recognition of domain I of β2GPI and β2GPI dimerization, and it was due to attenuated eNOS S1179 phosphorylation mediated by protein phosphatase 2A (PP2A). Furthermore, LDL receptor family member antagonism with receptor-associated protein (RAP) prevented aPL inhibition of eNOS in cell culture, and *ApoER2*$^{-/-}$ mice were protected from aPL inhibition of eNOS in vivo. Moreover, both aPL-induced increases in leukocyte–endothelial cell adhesion and thrombus formation were absent in eNOS$^{-/-}$ and in *ApoER2*$^{-/-}$ mice. Thus, aPL-induced leukocyte–endothelial cell adhesion and thrombosis are caused by eNOS antagonism, which is due to impaired S1179 phosphorylation mediated by β2GPI, apoER2, and PP2A. Our results suggest that novel therapies for APS can now be developed targeting these mechanisms.

Introduction

The antiphospholipid syndrome (APS) is an autoimmune disorder characterized by the presence of circulating antiphospholipid antibodies (aPL) and recurrent thrombosis (1). A link between APS and greater risk of atherosclerosis in peripheral and coronary arteries has also been established (2). aPL are directed not against phospholipids, but rather against plasma proteins with affinity for anionic cell surface phospholipids, and a pathogenetically important major subset of aPL is directed against β2-glycoprotein I (β2GPI) (3–7). Binding of aPL to phospholipid-bound β2GPI causes its dimerization, which further increases its affinity for negatively charged phospholipids and cell surfaces (8). The endothelium is a primary target of aPL, and pathogenic autoantibody binding to β2GPI causes the upregulation of adhesion molecule expression and a proinflammatory and prothrombotic endothelial cell phenotype (9). How aPL binding to β2GPI on the endothelial cell surface induces a transmembrane signal to modify endothelial cell behavior is unknown.

NO generated by the endothelial isoform of NOS (eNOS) is a key determinant of vascular health that regulates several physiological processes, including leukocyte adhesion, thrombosis, endothelial cell migration and proliferation, vascular permeability, and vascular smooth muscle cell growth and migration (10). The eNOS enzyme, which generates NO upon the conversion of L-arginine to L-citrulline, is activated by numerous extracellular stimuli and is promoted primarily by increases in the phosphorylation of S1179 (in bovine eNOS; S1177 in human eNOS) by PI3 kinase/Akt kinase and also by dephosphorylation of T497 (11–13). Whether aPL alter eNOS function is unknown.

To better understand the molecular basis of APS, we designed the present study to test the hypothesis that aPL-induced increases in leukocyte–endothelial cell adhesion and thrombus formation are caused by eNOS antagonism. In addition, we determined whether aPL-induced eNOS inhibition involves β2GPI, and if the process also requires an LDL receptor (LDLR) family member, particularly apoER2, which has the capacity to directly bind β2GPI (14, 15). Complementary experiments evaluating eNOS activation and leukocyte–endothelial cell adhesion were performed to link changes in enzyme activity with alterations in a key endothelial cell function that contributes to both the proinflammatory and the prothrombotic actions of aPL (16). Furthermore, the molecular underpinnings of eNOS antagonism by aPL were investigated

Authorship note: Sangeetha Ramesh and Craig N. Morrell contributed equally to this work. Philip W. Shaul and Chieko Mineo are co–senior authors.

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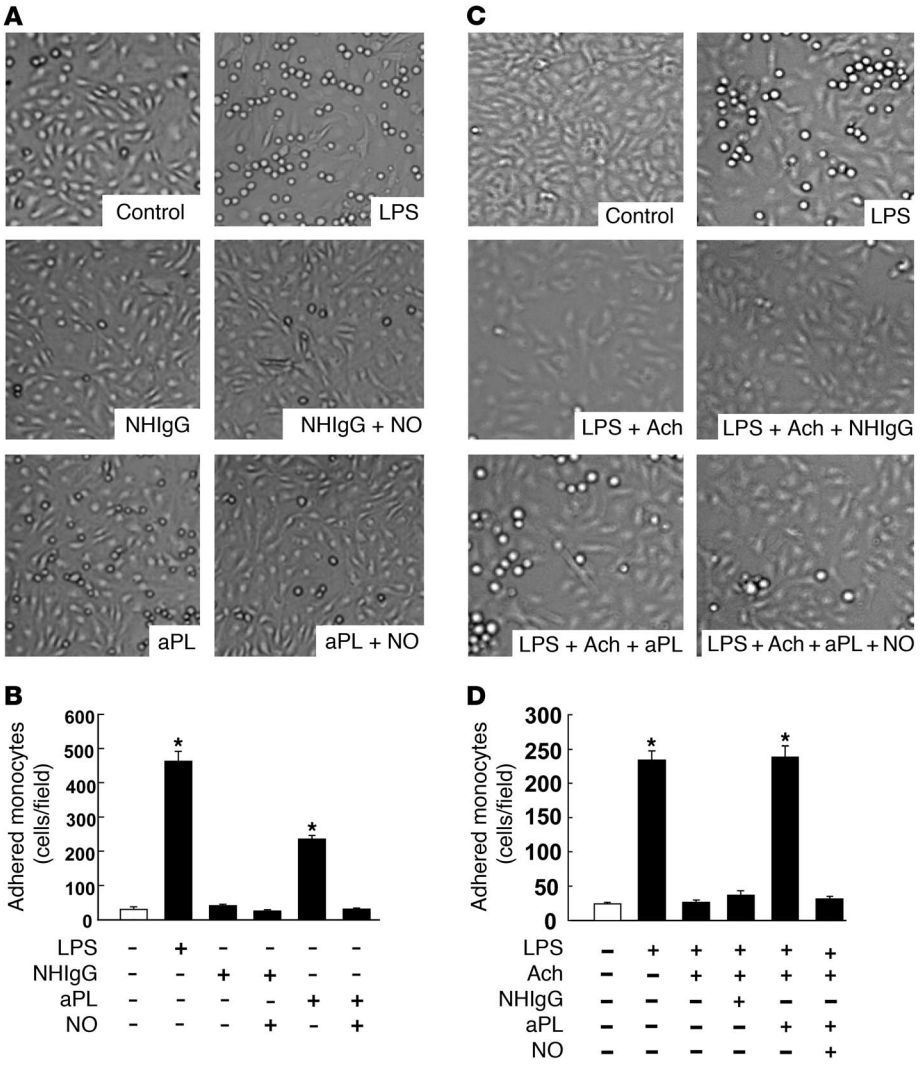

Figure 1
aPL-mediated increases in monocyte adhesion are due to decreased bioavailable NO. (**A**) Monocyte adhesion to BAECs was evaluated under the following conditions: untreated control, LPS (100 ng/ml), NHIgG (100 μg/ml), NHIgG + NO donor (SNAP, 20 μmol/l), aPL (100 μg/ml), and aPL + NO donor. (**B**) Graph representing summary data for **A**. (**C**) Monocyte adhesion to BAECs was evaluated under the following conditions: untreated control, LPS, LPS + Ach (10 μmol/k), LPS + Ach + NHIgG, LPS + Ach + aPL, and LPS + Ach + aPL + NO donor SNAP). (**D**) Graph representing summary data for **C**. In **B** and **D**, $n = 4$; $^*P < 0.05$ versus control. Original magnification, ×40 (**A** and **C**).

in studies of the mechanisms regulating eNOS phosphorylation and dephosphorylation.

Results

Increases in adhesion with aPL are due to decreased bioavailable NO. To begin to test the potential role of alterations in NO in the effects of aPL on endothelium, we performed studies of monocyte adhesion to bovine aortic endothelial cells (BAECs). Representative high-power-field images are shown in Figure 1A. Compared with control conditions, treatment of BAECs with LPS, used as a positive control, predictably caused an increase in monocyte adhesion. Whereas treatment of the endothelial cells with normal human IgG (NHIgG) obtained from healthy individuals had no effect, polyclonal aPL isolated from APS patients caused a marked increase in adhesion. The impact of aPL was fully reversed by addition of the NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP). Summary data from 3 independent experiments are shown in Figure 1B.

To further determine whether the aPL-induced increase in endothelial cell–monocyte interaction was due to the antagonism of NO production, we performed additional experiments with the eNOS agonist acetylcholine (Ach; Figure 1C). The increase in monocyte adhesion with LPS was predictably reversed by Ach. Whereas NHIgG had no effect on Ach attenuation of adhesion, polyclonal aPL completely prevented the blunting of adhesion by the eNOS agonist, and the impact of aPL on monocyte adhesion was fully reversed by the NO donor SNAP. Summary data from 3 independent experiments are shown in Figure 1D. These results indicate that aPL-induced increases in monocyte adhesion to endothelium are due to decreased bioavailable NO.

aPL antagonize eNOS. To directly determine whether aPL inhibit eNOS activation, we pretreated BAECs with NHIgG or polyclonal aPL and evaluated eNOS stimulation by VEGF (Figure 2A). Whereas VEGF caused a greater than a 2-fold increase in eNOS activity in NHIgG-treated cells, which is similar to the response in nontreated cells (17), polyclonal aPL caused a marked attenuation in eNOS activation. Identical findings were obtained in studies performed with human aortic endothelial cells (HAECs) (Figure 2B). To test the impact of aPL on eNOS activation by a different agonist and to prepare for in vivo experiments in mice, we performed studies with Ach in MFLM-91U mouse endothelial cells (Figure 2C). aPL blunted eNOS activation by Ach in the mouse endothelial cells. Thus, aPL attenuates eNOS activation by diverse agonists in bovine, human, and mouse endothelium.

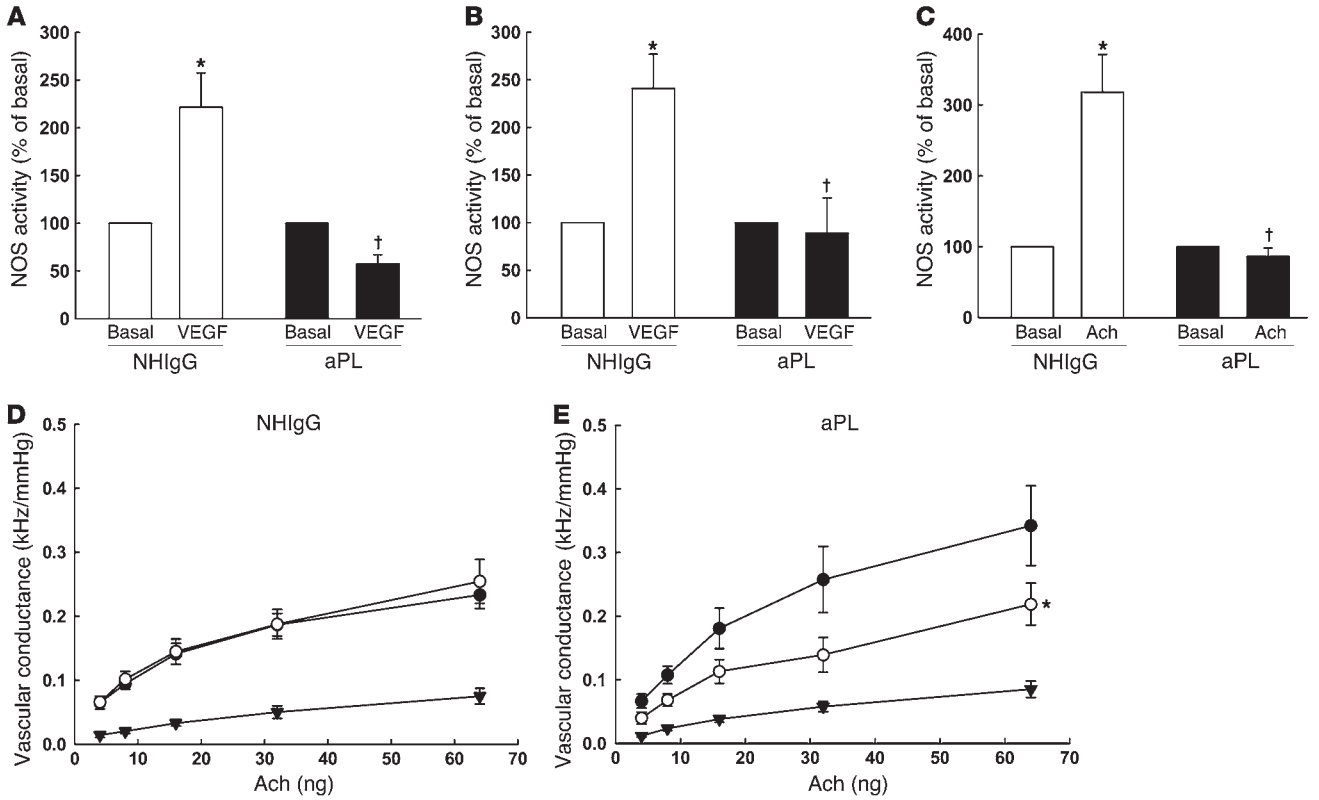

Figure 2

aPL antagonize eNOS. BAECs (**A**), HAECs (**B**), or MFLM-91U cells (**C**) were pretreated for 15 minutes with NHIgG or aPL (100 μg/ml), and eNOS activity was then measured under basal conditions or during stimulation with VEGF (100 ng/ml) or Ach (10 μmol/l) for 15 minutes. In **A**–**C**, $n = 4$; $^*P < 0.05$ versus basal, $^\dagger P < 0.05$ versus NHIgG. (**D** and **E**) Male C57BL/6 mice were instrumented, and changes in carotid vascular conductance, kHz/mmHg. in response to eNOS activation were compared in mice injected with NHIgG (**D**, 2 mg/mouse i.v.) and aPL (**E**, 2 mg/mouse i.v.). Dose responses to Ach were determined sequentially at baseline (filled circles), 60 minutes after administration of NHIgG or aPL (open circles), and 10 minutes after L-NAME administration (inverted triangles). In **D** and **E**, $n = 6$/group; $^*P < 0.05$ versus baseline (ANOVA).

To determine whether aPL antagonism of eNOS is operative in vivo, we measured Ach-mediated changes in carotid vascular conductance in C57BL/6 mice. Responses to Ach were sequentially evaluated at baseline, following NHIgG or polyclonal aPL administration, and following NOS antagonism with nitro-L-arginine methyl ester (L-NAME). Whereas the administration of NHIgG had no effect on Ach-mediated increases in vascular conductance (Figure 2D), aPL decreased the Ach response by 50% (Figure 2E). These findings demonstrate that the antagonism of eNOS by aPL observed in cultured endothelial cells also occurs in vivo.

eNOS antagonism and the resulting increase in adhesion with aPL are mediated by β2GPI. We first tested the role of β2GPI in eNOS antagonism by comparing the actions of polyclonal aPL with and without prior serum deprivation, which displaces β2GPI from the cell surface (18, 19). In serum-exposed endothelial cells, aPL caused robust inhibition of eNOS activation by VEGF (Figure 3A). In contrast, after serum deprivation for 24 hours, which caused a loss of β2GPI bound to the cells (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI39828DS1), aPL did not inhibit eNOS activity. To directly assess the role of β2GPI, we examined the effect on eNOS activation of FC1, a mouse monoclonal aPL reactive with β2GPI (20). Whereas treatment with control mouse IgG did not attenuate eNOS activation by VEGF, FC1 inhibited eNOS activation (Figure 3B). Since pathogenic aPL that recognize β2GPI are most often directed against domain I (21–27) and the epitope on β2GPI recognized by FC1 is unknown, experiments were then performed to compare the effects of a monoclonal antibody to domain I (3F8) versus a monoclonal antibody against domain II of β2GPI (2aG4). Similar to polyclonal aPL, 3F8 caused complete inhibition of eNOS activation by VEGF; in contrast, 2aG4 had no effect (Figure 3C). To delineate how β2GPI domain I–mediated eNOS antagonism impacts endothelial cell function, we performed monocyte adhesion experiments with BAECs treated with 3F8 (Figure 3D). Whereas the isotype-matched control IgG BBG had no effect on monocyte adhesion, 3F8 caused a marked increase in adhesion comparable to that observed with LPS, and treatment with the NO donor SNAP fully reversed the effect of 3F8 as well as that of LPS. To further confirm the role of β2GPI in the increased adhesion induced by the impairment of NO production, we performed additional studies with the eNOS agonist Ach (Figure 3E). Compared with control conditions, LPS caused an increase in monocyte adhesion, and this was reversed by Ach. Whereas NHIgG, BBG, and 2aG4 had no effect on Ach attenuation of adhesion, both polyclonal aPL and 3F8 directed against domain I of β2GPI completely prevented the blunting of adhesion by the eNOS agonist. To test whether eNOS antagonism by aPL may be due to the dimerization of β2GPI, we examined the effect of purified β2GPI dimers on eNOS activation (Figure 3F). Whereas

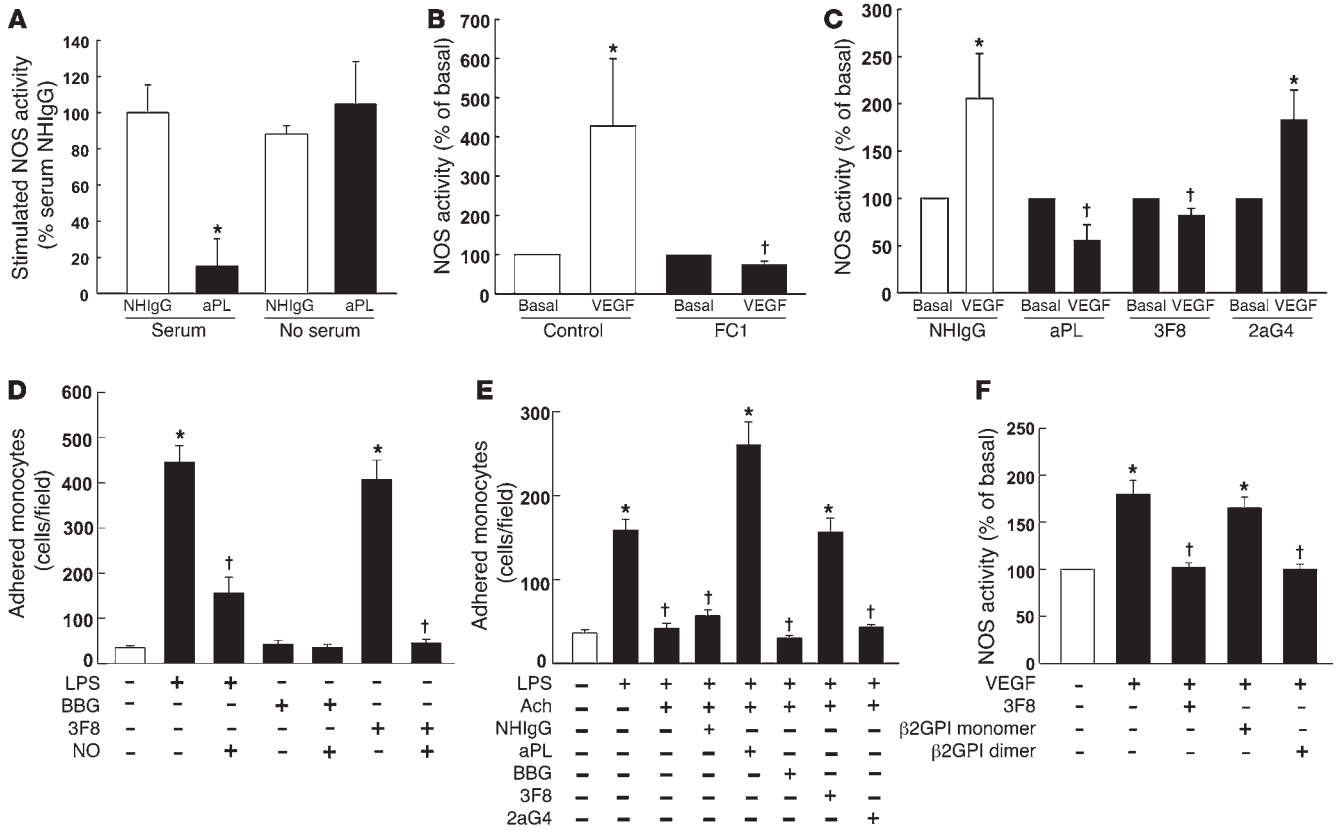

Figure 3

eNOS antagonism by aPL and resulting changes in monocyte adhesion require β2GPI. (**A**) BAECs cultured in the presence or absence of serum for 18 hours were pretreated with NHIgG or aPL (100 μg/ml, 15 minutes), and eNOS activity stimulated by VEGF (100 ng/ml, 15 minutes) was measured. $n = 4$; $^*P < 0.05$ versus no aPL. (**B**) BAECs were pretreated with control mouse IgG1 (10 μg/ml) or mouse monoclonal antibody to β2GPI (FC1, 10 μg/ml) for 15 minutes, and basal and VEGF-stimulated eNOS activity was measured. $n = 4$; $^*P < 0.05$ versus basal, $^\dagger P < 0.05$ versus control. (**C**) BAECs were pretreated with NHIgG or aPL, or antibodies to β2GPI (3F8 or 2aG4, 10 μg/ml) for 15 minutes, and basal and VEGF-stimulated eNOS activity was measured. $n = 6$, $^*P < 0.05$ versus basal, $^\dagger P < 0.05$ versus NHIgG. (**D**) BAECs were treated with vehicle (control) or LPS (100 ng/ml) with or without control IgG (BBG, 10 μg/ml) or 3F8, in the presence or absence of the NO donor SNAP (NO, 20 μmol/l) for 18 hours, and monocyte adhesion was evaluated. $n = 4$; $^*P < 0.05$ versus control, $^\dagger P < 0.05$ versus no NO treatment. (**E**) BAECs were treated with vehicle or LPS with or without Ach (10 μmol/l), in the presence of NHIgG, aPL, BBG, 3F8, or 2aG4, and monocyte adhesion was evaluated. $n = 4$; $^*P < 0.05$ versus no treatment, $^\dagger P < 0.05$ versus LPS alone. (**F**) BAECs were pretreated with 3F8, monomeric β2GPI (1.5 μg/ml), or β2GPI dimer (1.5 μg/ml) for 15 minutes, and basal and VEGF-stimulated eNOS activity was measured. $n = 6$; $^*P < 0.05$ versus basal, $^\dagger P < 0.05$ versus VEGF alone.

monomeric β2GPI had no effect on VEGF-induced eNOS activation, dimerized β2GPI completely inhibited eNOS, paralleling the effect of 3F8. These cumulative findings indicate that β2GPI mediates eNOS antagonism and the resulting increase in endothelial cell–monocyte adhesion caused by aPL, and that the underlying mechanism likely entails β2GPI dimerization.

Studies in mice suggest that there is an important role for complement in aPL-induced thrombosis and aPL-induced fetal loss (28–32). To determine whether complement participates in the proximal event of eNOS antagonism by aPL, we performed experiments evaluating eNOS activation in BAECs that were serum-exposed or maintained in heat-inactivated, complement-free serum (Supplemental Figure 2A). aPL-mediated eNOS antagonism occurred in a similar manner under both conditions. The role of complement was further assessed in studies of endothelial cell–monocyte adhesion induced by the anti–β2GPI domain I antibody 3F8 (Supplemental Figure 2B). 3F8 caused similar increases in adhesion in BAECs that were serum-exposed or maintained in heat-inactivated, complement-free serum. Thus, complement is not required for the effects of aPL on eNOS or for the resulting increase in endothelial cell–monocyte adhesion.

The contribution of Fc receptors to the pathogenicity of aPL is controversial. There is evidence suggesting that aPL may exert thrombogenic effects via the crosslinking of activating Fc receptors (FcγRIIA) and that polymorphisms in Fc receptor are a clinical predictor of relative thrombosis risk in APS (33, 34). However, studies in a mouse model of aPL-induced fetal loss have excluded a role for activating Fc receptors (30, 35). To determine whether Fc receptors are involved in aPL-mediated eNOS antagonism, we treated BAECs with NHIgG, intact polyclonal aPL, or bivalent $F(ab)'_2$ fragments of polyclonal aPL (Supplemental Figure 2C). Whereas VEGF activation of eNOS occurred in NHIgG-treated cells, intact aPL and bivalent $F(ab)'_2$ fragments of aPL antagonized eNOS activation to a comparable degree. Thus, the F(ab)′

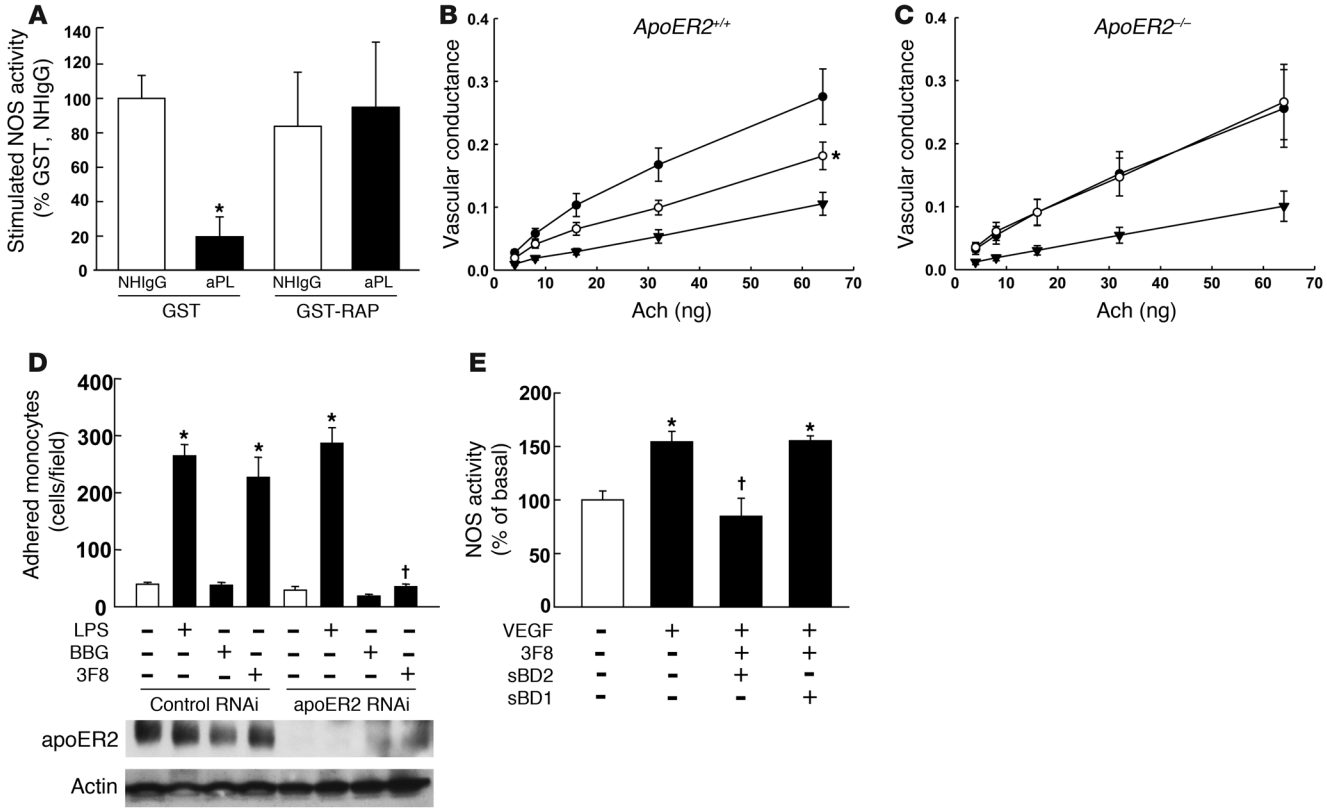

Figure 4

eNOS antagonism by aPL and resulting changes in monocyte adhesion require apoER2 and β2GPI-apoER2 interaction. (**A**) BAECs were pretreated with GST (12 μg/ml) or GST-RAP (GST, 12 μg/ml; RAP 30 μg/ml) and either NHIgG or aPL (100 μg/ml for 15 minutes), and basal and VEGF-stimulated (100 ng/ml) eNOS activity was measured. $n = 4$; $^*P < 0.05$ versus NHIgG. (**B** and **C**) *ApoER2*$^{+/+}$ and *ApoER2*$^{-/-}$ mice were instrumented, and changes in carotid vascular conductance in response to eNOS activation were compared before and after treatment with aPL (2 mg i.v.). Dose-responses to Ach were determined sequentially at baseline (filled circles), 60 minutes after aPL treatment (open circles), and 10 minutes after L-NAME administration (inverted triangles). $n = 6$/group; $^*P < 0.05$ versus baseline (ANOVA). (**D**) BAECs transfected with control (control RNAi) or double-stranded RNA targeting apoER2 were exposed to vehicle, LPS (100 ng/ml), control IgG (BBG, 10 μg/ml), or anti-β2GPI antibody (3F8, 10 μg/ml) for 18 hours, and monocyte adhesion was evaluated (upper panel). $n = 4$; $^*P < 0.05$ versus no treatment, $^\dagger P < 0.05$ versus control RNAi. In parallel studies, whole cell lysates were immunoblotted for apoER2 and actin (lower panel). (**E**) BAECs were pretreated with 3F8 (2 μg/ml) in the presence of peptide with sequence identical to the second LDL-binding domain (sBD2, 125 μg/ml) or the first LDL-binding domain of apoER2 (sBD1, 125 μg/ml) for 15 minutes, and basal and VEGF-stimulated eNOS activity was measured. $n = 6$; $^*P < 0.05$ versus basal, $^\dagger P < 0.05$ versus VEGF alone.

region of aPL is sufficient to antagonize eNOS and the Fc region is not required, indicating that Fc receptors are not required for the actions of aPL on eNOS.

eNOS antagonism and resulting increase in adhesion with aPL require apoER2. Since we found that aPL antagonism of eNOS is mediated by β2GPI and is mimicked by dimeric β2GPI, and since dimeric β2GPI has the capacity to bind directly to members of the LDLR family, including apoER2 and VLDLR (8, 14), we next studied the role of member(s) of the LDLR family using the inhibitor receptor-associated protein (RAP) (ref. 36 and Figure 4A). In cells pretreated with GST control, polyclonal aPL blunted eNOS activation. In contrast, in cells pretreated with GST-RAP, aPL did not inhibit eNOS activity. These findings indicate that member(s) of the LDLR family expressed in endothelial cells are required for aPL-mediated eNOS antagonism. To determine whether apoER2 is the transmembrane protein coupling aPL to eNOS, we assessed the effect of aPL on Ach-mediated increases in carotid vascular conductance in *ApoER2*$^{+/+}$ versus *ApoER2*$^{-/-}$ mice. Whereas the administration of aPL blunted Ach-induced increases in vascular conductance in *ApoER2*$^{+/+}$ mice (Figure 4B), aPL had no effect on the Ach response in *ApoER2*$^{-/-}$ mice (Figure 4C). To determine the requirement for apoER2 in direct aPL action on endothelial cells, we used siRNA to diminish expression of the receptor in BAECs and studied endothelial cell–monocyte adhesion (Figure 4D). In apoER2 RNAi–treated cells, apoER2 protein expression was decreased by more than 90% (Figure 4D). Whereas apoER2 knockdown had no effect on LPS-induced adhesion, 3F8-mediated adhesion was fully prevented.

Having found that aPL antagonism of eNOS and the resulting increase in endothelial cell–monocyte adhesion are mediated by both β2GPI and apoER2, we next examined the role of their interaction. In prior studies in isolated platelets, a soluble peptide based on the sequence of the first LDL-binding domain (BD1) of apoER2′, designated sBD1, prevented the interaction of domain V of β2GPI with the receptor (37, 38). We employed sBD1 in studies of eNOS antagonism by the anti-β2GPI antibody 3F8 in BAECs (Figure 4E), and found that

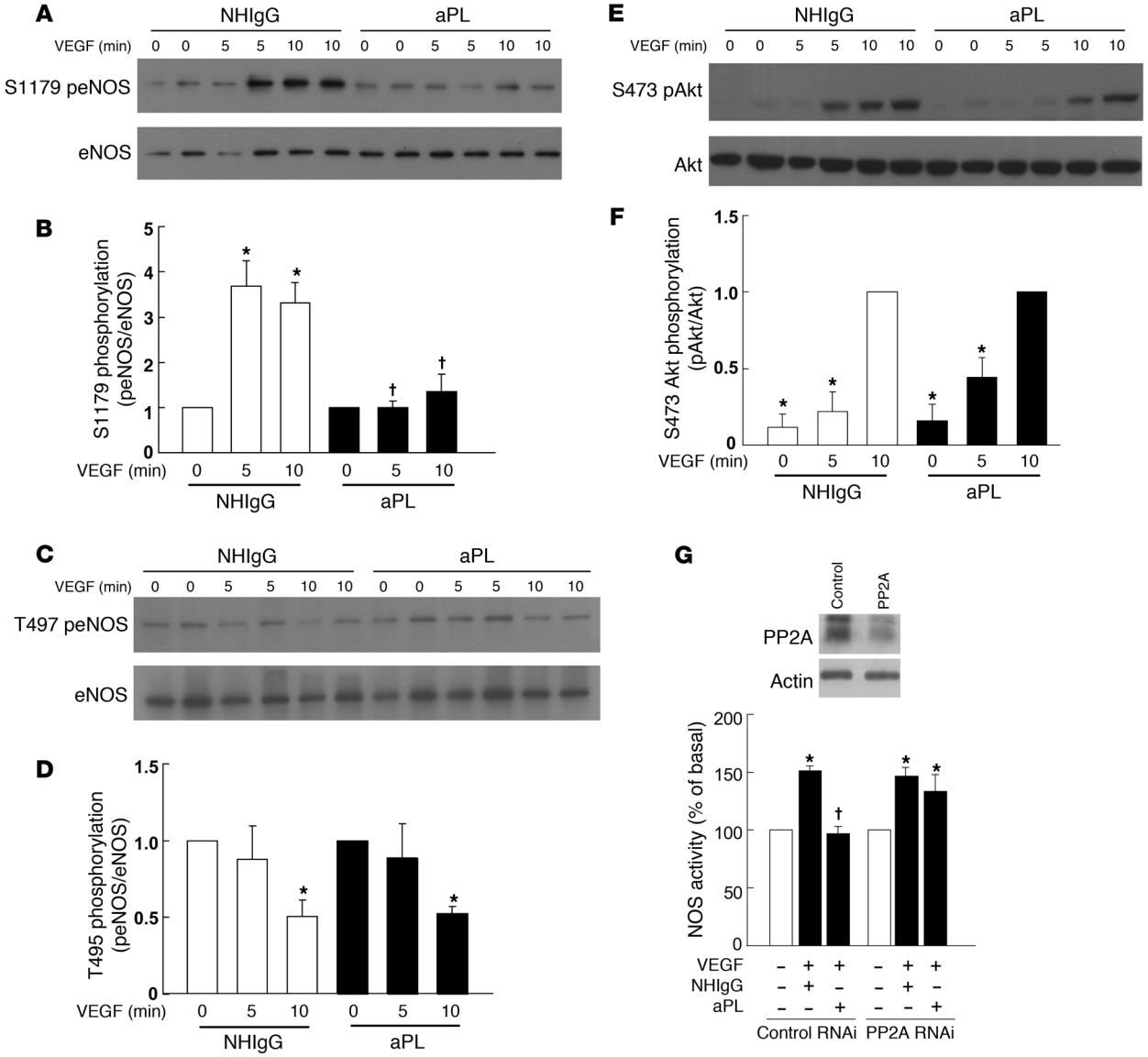

Figure 5

aPL blunts eNOS S1179 phosphorylation and enzyme activation via PP2A. (**A** and **B**) BAECs were serum-starved for 18 hours, incubated with β2GPI (5 μg/ml for 1 hour), pretreated with NHIgG or aPL (100 μg/ml for 15 minutes), and incubated with VEGF (100 ng/ml for 0, 5, 10 minutes). Whole cell lysates were prepared and immunoblotted for phospho-eNOS and total eNOS using polyclonal phospho-S1179 eNOS antibody and monoclonal total eNOS antibody, respectively. (**C** and **D**) Samples were also immunoblotted using phospho-T497 eNOS and total eNOS antibody. In **B** and **D**, summary values shown are mean ± SEM. $n = 6–8$; $^*P < 0.05$ versus time 0; $^\dagger P < 0.05$ versus NHIgG. (**E** and **F**) In parallel studies samples were immunoblotted for phospho-S473 Akt and total Akt. The abundance of phospho-Akt/total Akt is expressed relative to abundance at 10 minutes to accommodate a lack of detection of phospho-Akt in some samples in the absence of VEGF stimulation (time 0). In **F**, summary values shown are mean ± SEM. $n = 4$; $^*P < 0.05$ versus 10 minutes VEGF. (**G**) BAECs transfected with control (control RNAi) or double-stranded RNA targeting PP2A were serum-starved for 18 hours and incubated with β2GPI. The cells were further pretreated with NHIgG or aPL, and basal and VEGF-stimulated eNOS activity was measured. $n = 6$; $^*P < 0.05$ versus basal, $^\dagger P < 0.05$ versus NHIgG. Immunoblotting of whole cell lysates for PP2A and actin revealed approximately 50% knockdown of PP2A (inset).

whereas control peptide with the sequence of the second LDL-binding domain (sBD2) did not alter the antagonism of eNOS, sBD1 fully prevented the inhibition. Thus, apoER2 and interaction between BD1 of the receptor and domain V of β2GPI are required for aPL antagonism of eNOS and the resulting increase in monocyte adhesion.

aPL blunts eNOS S1179 phosphorylation via PP2A. The basis for aPL antagonism of eNOS was next determined in studies in BAECs of the processes regulating the phosphorylation and dephosphorylation of the enzyme at S1179 (S1177 in human eNOS) and T497 (11, 12, 39). In initial experiments evaluating the degree of eNOS phosphorylation, the cells were serum-starved for 24 hours to deplete β2GPI. Following serum starvation, neither VEGF-induced S1179 phosphorylation nor VEGF-induced T497 dephosphorylation was altered by polyclonal aPL (Supplemental Figure 3), paral-

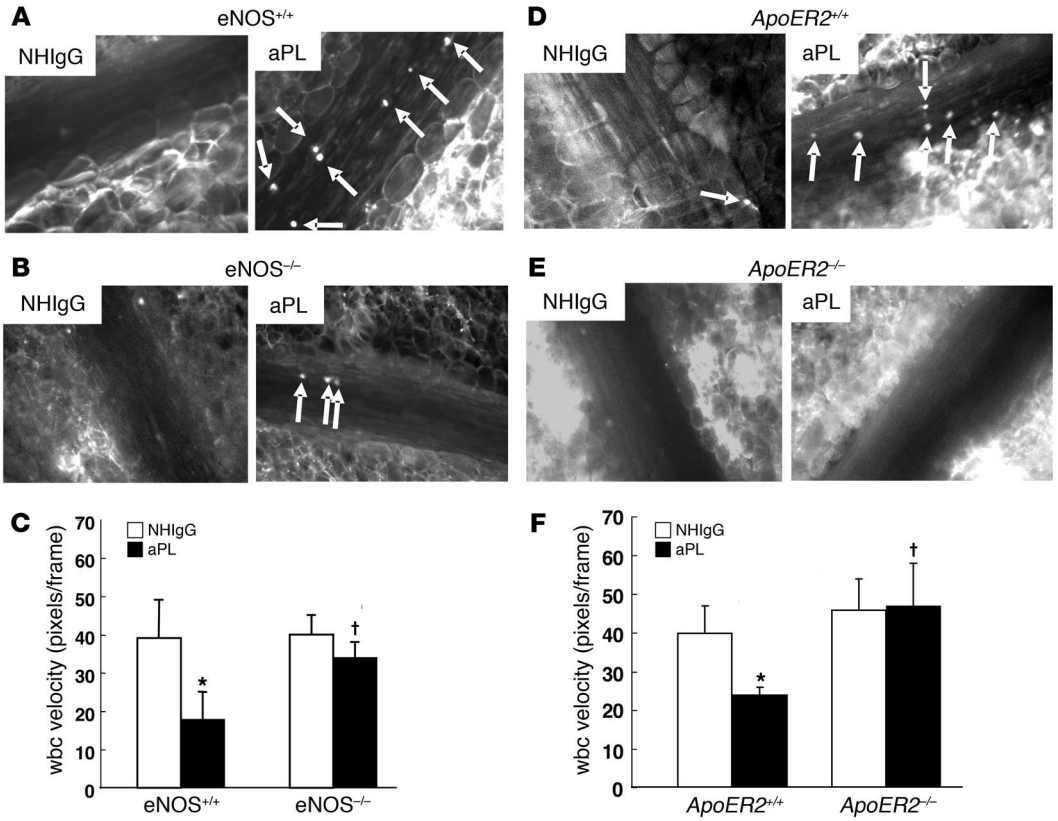

Figure 6

aPL-induced leukocyte–endothelial cell adhesion in mice is mediated by eNOS and apoER2. *eNOS*$^{+/+}$ (**A**) and *eNOS*$^{-/-}$ (**B**) mice were i.p. injected with NHIgG or aPL (100 μg) and 24 hours later prepared for intravital microscopy. Leukocytes were fluorescence-labeled by injection of rhodamine 6G, and the mesentery was exposed for the observation and recording of images of leukocyte adhesion and rolling. Representative still images are shown (leukocytes indicated by arrows). (**C**) Velocity of leukocyte (wbc) rolling in *eNOS*$^{+/+}$ and *eNOS*$^{-/-}$ mice. Parallel experiments were performed in *ApoER2*$^{+/+}$ (**D**) and *ApoER2*$^{-/-}$ (**E**) mice, and representative still images are shown. (**F**) Velocity of leukocyte (wbc) rolling in *ApoER2*$^{+/+}$ and *ApoER2*$^{-/-}$ mice. In **C** and **F**, values are mean ± SEM. n = 5–7/group; $^{*}P < 0.05$ vs NHIgG, $^{\dagger}P < 0.05$ versus *eNOS*$^{+/+}$ or *ApoER2*$^{+/+}$.

leling the lack of eNOS antagonism by aPL after serum starvation (Figure 3A). However, following reconstitution with recombinant human β2GPI (18, 19) (Supplemental Figure 1), aPL caused full inhibition of VEGF-induced S1179 phosphorylation (Figure 5, A and B); VEGF-induced T497 dephosphorylation was unaffected (Figure 5, C and D). To elucidate the basis for blunted eNOS s1179 phosphorylation caused by aPL, potential changes in Akt activation were then interrogated in serum-starved cells following reconstitution with β2GPI. Whereas polyclonal aPL treatment blunted eNOS S1179 phosphorylation in response to VEGF (Figure 5, A and B), Akt phosphorylation at S473, indicative of activation of the kinase, was unaffected (Figure 5, E and F). Thus, aPL antagonize eNOS activity by attenuating the phosphorylation of S1179, and the actions of aPL occur distal to Akt.

In numerous paradigms, eNOS S1179 is dephosphorylated and eNOS enzymatic activity is consequently decreased by the phosphatase PP2A (39, 40). We therefore assessed the role of PP2A in aPL-induced changes in eNOS activity by siRNA knockdown. PP2A knockdown of approximately 50% (Figure 5G, inset) prevented aPL antagonism of eNOS activation by VEGF (Figure 5G). Thus, aPL antagonize eNOS by attenuating S1179 phosphorylation via the activation of the phosphatase PP2A.

aPL-induced leukocyte–endothelial cell adhesion in vivo is mediated by eNOS and apoER2. Having demonstrated in cultured endothelial cells that aPL antagonize eNOS activity resulting in increased monocyte–endothelial cell adhesion, we determined whether these mechanisms are operative in vivo in studies of leukocyte–endothelial cell adhesion in the mouse mesenteric microcirculation. Under the control conditions present with NHIgG treatment, leukocyte adhesion (still images are shown in Figure 6, A and B) and wbc velocity (Figure 6C) were similar in *eNOS*$^{+/+}$ and *eNOS*$^{-/-}$ mice. Despite the difference in endothelium-derived NO in these mice, similar leukocyte–endothelial cell adhesion has been observed in prior studies at baseline, and this has been attributed to compensatory mechanisms (41). As expected, aPL administration to *eNOS*$^{+/+}$ mice caused an increase in leukocyte–endothelial cell interaction (Figure 6A), and an associated decrease in leukocyte velocity (Figure 6C). In contrast, in *eNOS*$^{-/-}$ mouse aPL had minimal effect on leukocyte interaction with endothelium (Figure 6B) and therefore did not alter leukocyte velocity (Figure 6C). Videos of the intravital fluorescence microscopy are provided in Supplemental Videos 1–4. These results indicate that eNOS is critical to aPL-induced increases in leukocyte adhesion to vascular endothelium in vivo.

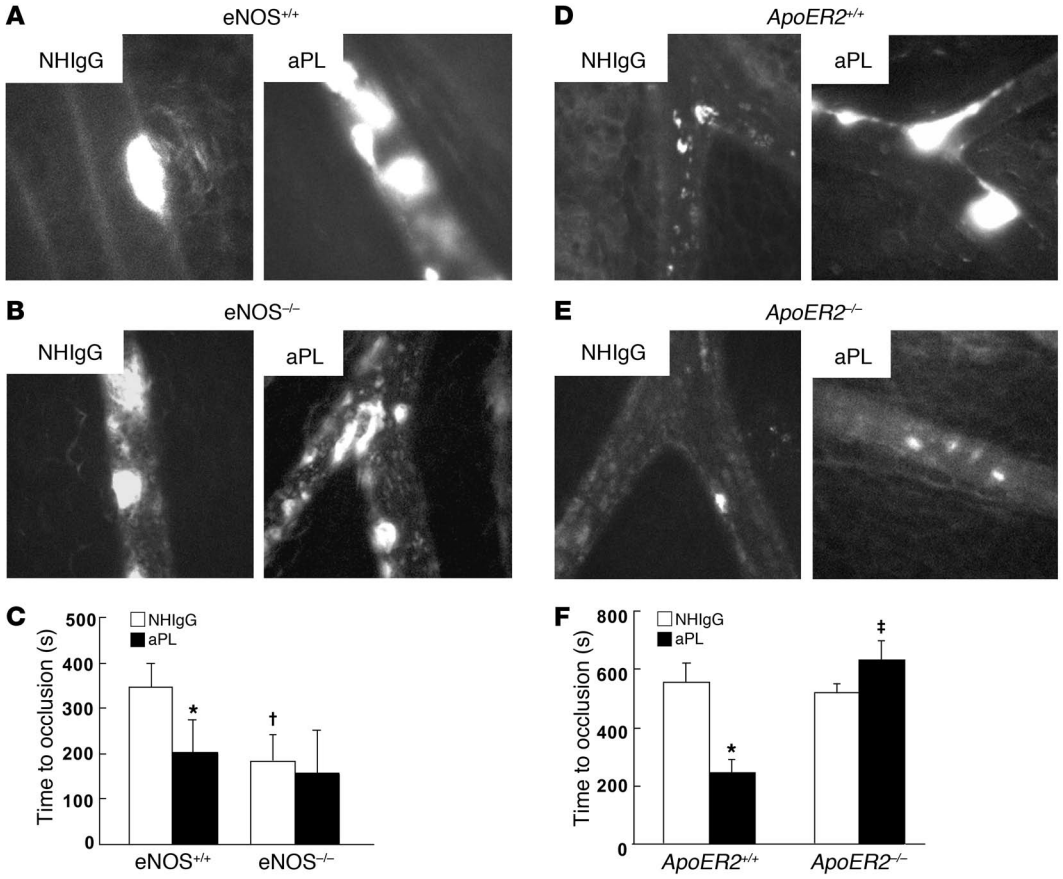

Figure 7

aPL-induced thrombosis in mice is mediated by eNOS and apoER2. *eNOS*$^{+/+}$ (**A**) and *eNOS*$^{-/-}$ (**B**) mice were i.p. injected with NHIgG or aPL (100 μg) and 24 hours later prepared for intravital microscopy. Platelets were fluorescence-labeled by injection of anti–mouse GPIbβ antibody, and the mesentery was exposed for the observation and recording of images of thrombi. Representative still images at 300 seconds are shown. (**C**) Time required for complete vessel occlusion in *eNOS*$^{+/+}$ and *eNOS*$^{-/-}$ mice. Parallel experiments were performed in *ApoER2*$^{+/+}$ (**D**) and *ApoER2*$^{-/-}$ (**E**) mice, and representative still images at 300 seconds are shown. (**F**) Time required for complete vessel occlusion in *ApoER2*$^{+/+}$ and *ApoER2*$^{-/-}$ mice. In **C** and **F**, values are mean ± SEM. n = 5–7/group; $^{*}P < 0.05$ versus NHIgG, $^{\dagger}P < 0.05$ versus *eNOS*$^{+/+}$, $^{\ddagger}P < 0.05$ versus *ApoER2*$^{+/+}$.

Having demonstrated in cell culture that both aPL-induced eNOS antagonism and the resulting increase in adhesion require apoER2, we investigated the role of apoER2 in vivo. In *ApoER2*$^{+/+}$ mice, aPL caused an increase in leukocyte–endothelial cell interaction (Figure 6D), resulting in decreased leukocyte velocity (Figure 6F). In contrast, in *ApoER2*$^{-/-}$ mice aPL had no effect on leukocyte interaction with endothelium (Figure 6E) and therefore did not change leukocyte velocity (Figure 6F). Videos of the intravital fluorescence microscopy are provided in Supplement Videos 5–7. Thus, aPL-induced increases in leukocyte–endothelial cell adhesion in vivo require apoER2.

aPL-induced thrombosis is mediated by eNOS and apoER2. Prior studies have demonstrated that aPL-induced changes in endothelial cell adhesion play a major role in the thrombosis invoked by the antibodies (16). In addition, it is well established that NO derived from eNOS attenuates both leukocyte–endothelial cell interactions and thrombosis (42–46). We therefore determined whether eNOS antagonism underlies aPL-induced thrombus formation in studies of *eNOS*$^{+/+}$ and *eNOS*$^{-/-}$ mice (Figure 7, A–C). As previously observed (42, 47), under the control conditions present with NHIgG treatment, the time to vessel occlusion was reduced in *eNOS*$^{-/-}$ versus *eNOS*$^{+/+}$ mice (Figure 7C). With aPL treatment of *eNOS*$^{+/+}$ mice, the time to vessel occlusion compared with NHIgG was shortened, indicating enhanced thrombus formation (Figure 7A, which displays still images at 300 seconds, and Figure 7C). In contrast, aPL did not alter thrombosis or change the time to occlusion in *eNOS*$^{-/-}$ mice (Figure 7, B and C).

The role of apoER2 in aPL-induced thrombosis was then investigated. In *ApoER2*$^{+/+}$ mice, aPL enhanced thrombus formation (Figure 7D), resulting in a decrease in the time to total vessel occlusion (Figure 7F). In contrast, in *ApoER2*$^{-/-}$ mice aPL had no effect on thrombus formation (Figure 7E) and therefore did not change the time to occlusion (Figure 7F). Thus, in addition to mediating aPL-induced increases in endothelial cell–leukocyte adhesion, apoER2 and eNOS are also critically involved in aPL-induced thrombosis.

Discussion

In the present study we have demonstrated that aPL inhibit the activation of eNOS and that the resulting decline in NO production underlies the promotion of leukocyte–endothelial cell adhesion

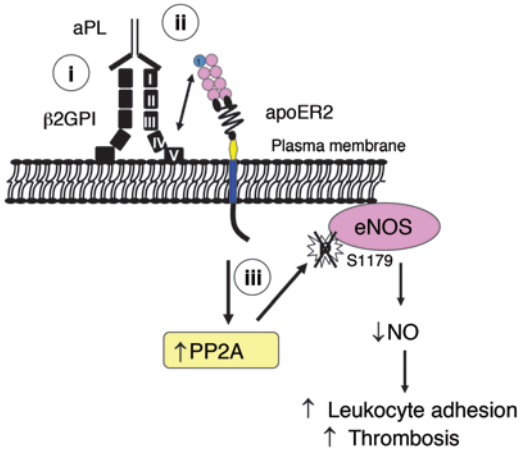

Figure 8
aPL promote leukocyte adhesion and thrombosis by antagonizing eNOS via β2GPI, apoER2, and the phosphatase PP2A. (i) aPL binding to domain I of β2GPI causes β2GPI dimerization and (ii) interaction between domain V of β2GPI and the first LDL-binding domain of apoER2 (BD1). Through yet-to-be-determined mechanism(s), the interaction of β2GPI with apoER2 causes (iii) increased activation of PP2A. This promotes the dephosphorylation of S1179 of eNOS, yielding decreased enzymatic activity and a decline in bioavailable NO, which results in increased leukocyte adhesion and increased thrombosis.

and thrombus formation by aPL (summary in Figure 8). Considering the primary role of eNOS-derived NO in both the anti-adhesive and antithrombotic characteristics of healthy endothelium (10), eNOS antagonism by aPL is likely to be a critical initiating process in the pathogenesis of the vascular manifestations of APS.

We found that aPL antagonize eNOS activity in cultured endothelial cells derived from multiple species, including humans. Studies of carotid vascular conductance in mice also showed that aPL attenuate eNOS activation, demonstrating the physiologic importance of this effect in vivo. These observations are consistent with the finding in APS patients that endothelium-dependent, NO-dependent flow-mediated dilation of the brachial artery is impaired, whereas endothelium-independent vasodilation is normal (48, 49). In addition, in APS patients there is a negative correlation between flow-mediated dilation and circulating levels of the adhesion molecules VCAM-1 and ICAM-1 (48). Consistent with this inverse relationship between the capacity for NO production and the degree of vascular inflammation in APS patients, our work in both cultured endothelial cells and in vivo in mice now reveals a causal link between aPL-induced eNOS antagonism and increased leukocyte–endothelial cell adhesion.

To define the underlying mechanisms, we examined the role of β2GPI. Using both loss-of-function and gain-of-function strategies, we determined that β2GPI mediates eNOS antagonism by aPL. Furthermore, monoclonal Ab to domain I of β2GPI, which is the domain primarily targeted by pathogenic aPL that recognize β2GPI (7, 21, 22, 24, 26, 27, 50), and not Ab to domain II, caused eNOS antagonism and the resulting increase in adhesion (Figure 8, i). Moreover, purified dimeric β2GPI, but not monomeric β2GPI, inhibited eNOS activation. Thus, we have demonstrated that the recognition of domain I of β2GPI and its dimerization are the upstream events in aPL antagonism of eNOS and its consequences. We further discovered that the downstream process leading to eNOS inhibition is attenuated eNOS S1179 phosphorylation caused by the activation of the phosphatase PP2A (Figure 8, iii).

There is considerable evidence that complement activation plays a role in the ultimate manifestations of APS. The inhibition of the complement cascade by the C3 convertase inhibitor complement receptor 1–related gene protein y–Ig (Crry-Ig) or the administration of anti-C5 monoclonal antibody reverses aPL-mediated thrombosis in mice, and C3- and C5-null mice are resistant to the effects of aPL (51). Complement activation also participates in aPL-induced fetal loss during pregnancy (28). Our finding that aPL-mediated eNOS inhibition does not require complement is consistent with the recent proposal that APS pathogenesis entails initial direct effects of aPL on endothelium, and possibly also on platelets, which are then amplified by the ensuing activation of complement via mediators such as C3a and C5a (51). Our elucidation of the proximal mechanisms responsible for aPL effects on endothelium now makes it possible to test potential cause-effect linkage between endothelial aPL action and complement activation.

To date, the basis by which pathogenic aPL recognition of β2GPI on the cell surface elicits a transmembrane signal to modify intracellular events in endothelium has been poorly understood (52–54). In biochemical analyses, it has been demonstrated that β2GPI binds directly to LDLR family members including apoER2, VLDLR, LRP, and megalin (14), and apoER2 and VLDLR are expressed in endothelium (55, 56). Using RAP in cultured endothelial cells, we first showed that an LDLR family protein is required for aPL-mediated eNOS inhibition. Then in in vivo studies, we identified that receptor to be apoER2. We provided further evidence of the requirement for apoER2 in studies of aPL-induced endothelial cell–monocyte adhesion entailing knockdown of the receptor in endothelium by RNAi. Moreover, by using a recombinant soluble form of LDL-binding domain 1 of ApoER2 (sBD1) that inhibits dimeric β2GPI binding via domain V to apoER2 (38, 57), we showed that interaction between β2GPI and the receptor mediates aPL actions in endothelium (Figure 8, ii). We then expanded on these numerous in vitro and ex vivo findings in studies of leukocyte–endothelial cell adhesion and thrombus formation in wild-type, *eNOS*$^{-/-}$, and *ApoER2*$^{-/-}$ mice. Although the results for aPL-induced thrombosis in eNOS$^{+/+}$ versus *eNOS*$^{-/-}$ mice should be interpreted conservatively because of the differences in thrombosis under control conditions, the cumulative findings indicate that apoER2 and eNOS are important linchpins in aPL vascular actions in vivo. Thus, the basis for transmembrane signaling induced by aPL binding to extracellular β2GPI in endothelium has now been identified, and the molecular events by which apoER2 initiates intracellular processes in endothelium including PP2A activation can now be investigated.

Although direct actions of aPL on platelets are likely contributory to APS-related thrombosis, there is a major role for the activation of endothelial cell adhesion in aPL-induced thrombus formation (9, 58, 59). This has been most clearly indicated by studies demonstrating that *ICAM1*$^{-/-}$ mice and mice administered anti–VCAM-1 monoclonal antibody are fully protected from aPL-induced thrombus formation (16). However, the presence of aPL and aPL actions on endothelium are not sufficient, because most of the time patients with circulating aPL do not have thromboses. A "two-hit hypothesis" has been proposed in which aPL (the first hit) induces a threshold cellular perturbation in endothelium, or possibly in platelets, and another condition (the second hit) is required to trigger actual clot formation (60). In most prior stud-

ies of APS-related thrombosis in rodents, as well as in our experiments, thrombus formation must be induced (16). The second hit may involve a proinflammatory stimulus, since rats administered aPL with anti-β2GPI activity have spontaneous thromboses if they also receive LPS (61). These observations have led to the suggestion that infectious agents may play a dual role in APS pathogenesis by initially triggering the generation of cross-reactive anti-β2GPI antibodies (first hit) due to considerable amino acid sequence homology between β2GPI and a number of bacterial and viral components, and then by inducing an inflammatory response that serves as the second hit (62). With the molecular underpinning of the first hit now in hand, the nature of possible second-hit conditions can be better interrogated.

The proximal processes by which aPL cause changes in endothelial cell behavior have been elusive (52–54, 63). We now provide multiple lines of evidence demonstrating that aPL-induced increases in leukocyte–endothelial cell adhesion and thrombus formation are caused by eNOS antagonism, which is due to impaired S1179 phosphorylation mediated by β2GPI, apoER2, and PP2A. The identification of these molecules and the elucidation of how they interplay to invoke the endothelial actions of aPL and their sequelae now provide the framework for the development of new strategies to combat APS. With the new knowledge gained, it is now possible that the lifelong requirement for anticoagulation in the APS patient will be replaced by a mechanism-based therapy offering both far fewer complications and greater efficacy against this often devastating condition.

Methods

Animal models. In vivo studies were performed in male C57BL/6 mice, *ApoER2*$^{+/+}$ and *ApoER2*$^{-/-}$ littermates (129SvEv × C57BL/6J), and *eNOS*$^{+/+}$ and *eNOS*$^{-/-}$ littermates (C57BL/6). All animal experiments were approved by the Institutional Animal Care and Utilization Committees at the University of Texas Southwestern Medical Center, the Johns Hopkins University School of Medicine, and the University of Rochester School of Medicine and Dentistry. (Some experiments were performed at the Johns Hopkins University School of Medicine under C.N. Morrell.)

Cell culture and transfection. Primary BAECs were isolated and studied using previously described methods (64, 65), and HAECs (Cambrex Corp.) were cultured in EGM2 media (Cambrex Corp.) and used within 3–7 passages. MFLM-91U mouse endothelial cells were provided by Ann Akeson (Children's Medical Center, Cincinnati, Ohio, USA) and cultured in Ultraculture Media (Cambrex Corp.) (66, 67). U937 monocytes (human histiocytic lymphoma, ATCC) were grown in RPMI 1640 Medium (Sigma-Aldrich) containing 10% FBS. In siRNA experiments, BAECs were transfected with siRNAs using LipofectAMINE 2000 (Invitrogen) and studied 48 hours later. Double-stranded RNA with sequence 5′-CCAAGCUGCAAUCAUGGAA-3′ or 5′-ACUGGAAGCGGAAGAAUAC-3′ was designed to target the open reading frame of the bovine PP2A catalytic subunit Cα (accession number M16968) (68) or bovine apoER2 (accession number XM_865091), respectively, and control dsRNA was purchased from Dharmacon (catalog D-001210-01-20).

Antibody preparation. NHIgG was obtained from healthy non-autoimmune individuals. Polyclonal aPL were isolated from patients with APS, which were characterized as having high-titer aPL Ab (>140 GPL U), thromboses, and/or pregnancy losses (32). The relevant clinical and laboratory features of the patients who provided aPL are given in Supplemental Table 1. Individuals provided informed consent before participating in these studies. All protocols were approved by the Institutional Review Board of Hospital for Special Surgery. The IgGs were purified by affinity chromatography using protein G–Sepharose chromatography columns (Amersham Biosciences) (31, 32). Endotoxin was removed using Centriprep ultracentrifugation devices (Millipore), and lack of endotoxin was confirmed using the *Limulus* amebocyte lysate assay (30). Bivalent $F(ab')_2$ fragments of polyclonal aPL were prepared by digestion of purified aPL IgG pooled from patients using immobilized pepsin (Pierce Chemical Co.). The digested supernatants were passed through protein G–Sepharose to remove remaining intact IgG. Their purity was assessed by Western blot analysis using an antibody specific for the $F(ab')_2$ fragments, and their aPL reactivity was demonstrated by ELISA (30). Mouse monoclonal aPL FC1 (IgG1) obtained from NZW × BXSB F_1 mice that recognizes β2GPI was provided by Marc Monestier (Temple University, Philadelphia, Pennsylvania, USA) (20, 27). FC1 was purified from hybridoma supernatant by affinity chromatography on recombinant protein G–agarose. Matching mouse monoclonal IgG1 isotype control mAb was obtained from Sigma-Aldrich. Mouse monoclonal antibodies directed to either domain I or domain II of β2GPI (3F8 and 2aG4, respectively) and the control mouse monoclonal antibody BBG3 were prepared as previously described (69, 70).

Monocyte adhesion assay. The adhesion of U937 monocytes to monolayers of BAECs was evaluated as described previously (17). Confluent BAECs were exposed to aPL (100 μg/ml), NHIgG (100 μg/ml), anti-β2GPI mouse monoclonal antibody (3F8 or 2aG4, 10 μg/ml), control mouse monoclonal antibody BBG3 or subtype-matched control mouse IgG (10 μg/ml), or LPS (Sigma-Aldrich, 100 ng/ml) for 18 hours. In selected experiments, BAECs were exposed to LPS in the absence or presence of the eNOS agonist Ach (10 μmol/l) and either NHIgG or aPL (100 μg/ml). In additional studies, cells were also treated with the NO donor SNAP (20 μmol/l). Subsequently, BAECs were washed with PBS, U937 monocytes (1×10^6cells/dish) were added and incubated with BAEC under rotating conditions (benchtop incubator at 63 rpm) at 21°C for 15 minutes, nonadhering cells were removed by gentle washing with PBS, cells were fixed with 1% paraformaldehyde, and the number of adherent cells was determined in triplicate per ×40 magnification field.

eNOS activation assay. eNOS activation was assessed in intact endothelial cells by measuring the conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline (64). Cells were preincubated with NHIgG or aPL (100 μg/ml), and eNOS activity was then determined over 15 minutes in the continued presence of NHIgG or aPL, in the absence (basal) or presence of VEGF (2.4 pmol/l, 100 ng/ml) or Ach (10 μmol/l). In selected experiments, the cells were treated with anti-β2GPI monoclonal antibody (FC1, 3F8, or 2aG4; 10 μg/ml). Additional experiments were performed in cells pretreated with recombinant monomeric β2GPI (apple 2-β2GPI, 1.5 μg/ml) or dimerized β2GPI (apple 4-β2GPI, 1.5 μg/ml), which were was constructed and expressed as described previously (15, 38). Studies were also done to assess the role of LDLR family members in cells pretreated with GST-RAP or GST control (12 μg/ml for 15 minutes), which were purified as previously reported, and purity was confirmed by SDS-PAGE (36). The role of β2GPI-apoER2 interaction was determined using recombinant soluble LDL-binding domain 1 of apoER2 (sBD1, 125 μg/ml) or the control peptide representing LDL-binding domain 2 (sBD2) (37). Separate sets of experiments were done in BAECs treated with RNAi targeted to knockdown apoER2 or PP2A. Findings were replicated in 3 or more independent experiments.

Carotid vascular conductance. To determine whether aPL alters eNOS activity in vivo, we measured Ach-mediated increases in carotid vascular conductance before and after NHIgG or aPL administration to 12- to 18-week-old male mice (17). Mice were anesthetized with tiletamine (4 mg/kg), zolazepam (4 mg/kg), and xylazine (20 mg/kg) given i.p., catheters were placed in the right jugular vein and common carotid artery, and the mice were switched to inhalation anesthesia (1% isoflurane). A Doppler ultrasound flow probe (Crystal Biotech) was placed around the left common carotid artery, and

arterial pressure, carotid blood flow velocity, and calculated carotid vascular conductance (mean blood flow velocity/mean arterial pressure) were monitored and recorded (MacLab, AD Instruments). Vasodilator responses to i.v. injections of Ach (4–64 ng in a volume of 1–16 μl) were evaluated sequentially at baseline, 60 minutes after administration of NHIgG or aPL (2 mg i.v.), and 10 minutes after treatment with L-NAME (10 mg/kg i.v.).

Immunoblot analyses. Immunoblot analyses were performed to assess eNOS and Akt phosphorylation using polyclonal anti–phospho-S1179 eNOS antibody, polyclonal anti–phospho-T497 eNOS antibody, and polyclonal anti–phospho-S473 Akt antibody (Cell Signaling Technology). Total eNOS and Akt abundance was assessed using eNOS monoclonal antibody (BD Biosciences — Pharmingen) and Akt polyclonal antibody (Cell Signaling Technology). BAECs were placed in serum-free, phenol red–free DMEM overnight, human β2GPI (5 μg/ml; Haematologic Technologies Inc.) was added for 1 hour, and cells were preincubated with NHIgG or aPL (100 μg/ml) and treated with VEGF (2.4 pmol/l, 100 ng/ml) for 0–10 minutes. Findings were confirmed in 3 independent experiments. The loss of media-derived bovine β2GPI with serum starvation and the reconstitution with human β2GPI were confirmed by immunoblotting with monoclonal antibody to β2GPI (2aG4) (69, 70). Selected sets of immunoblot analyses were performed to assess the expression of apoER2 or PP2A after RNAi knockdown using anti-apoER2 rabbit polyclonal antibody (71) or anti-PP2A mouse monoclonal antibody (Millipore).

Leukocyte adhesion and thrombus formation in vivo. Leukocyte adhesion was determined as described previously (72). Briefly, male mice (5–8 weeks old) were i.p. injected with NHIgG or aPL (100 μg) and 24 hours later prepared for intravital microscopy. All leukocytes were fluorescence-labeled by injection of rhodamine 6G (100 μl of 0.05% solution i.v.), and the mesentery was exposed for the observation and recording of images of leukocyte adhesion and rolling using a Retiga digital camera (×200 magnification; QImaging). The velocity of leukocyte rolling was calculated by measuring the distance leukocytes traveled between frames. It should be noted that velocity was found to be similar in control (NHIgG-treated) $eNOS^{+/+}$ versus $eNOS^{-/-}$ mice, as has been observed for baseline adhesion to $eNOS^{+/+}$ versus $eNOS^{-/-}$ endothelium in some but not all prior reports (41, 73, 74). Thrombus formation was assessed as described previously (47, 75). Briefly, 24 hours following i.p. injection with NHIgG or aPL (100 μg/mouse), mice were i.v. injected with fluorescence-labeled anti–mouse GPIbβ antibody, which targets the platelet GPIb-V-IX complex. Then the mesenteric microcirculation was exteriorized, and thrombosis was induced by ferric chloride (10%, on Whatman filter paper for 45 seconds). Thrombus formation in arterioles was documented by capturing images (×200 magnification) every 1 second until total vessel occlusion occurred.

Statistics. All data are expressed as mean ± SEM. Two-tailed Student's *t* test or ANOVA was used to assess differences between 2 groups or among more than 2 groups, respectively, with Newman-Keuls post-hoc testing following ANOVA. *P* values less than 0.05 were considered significant.

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