Antiphospholipid Antibodies Attenuate Endothelial Repair and Promote Neointima Formation in Mice

Victoria Ulrich, PhD; Eddy S. Konaniah, BS; Wan-Ru Lee, PhD; Sadiksha Khadka, BS; Yu-Min Shen, MD; Joachim Herz, MD; Jane E. Salmon, MD; David Y. Hui, PhD; Philip W. Shaul, MD; Chieko Mineo, PhD

Background—Antiphospholipid syndrome patients have antiphospholipid antibodies (aPLs) that promote thrombosis, and they have increased cardiovascular disease risk. Although the basis for the thrombosis has been well delineated, it is not known why antiphospholipid syndrome patients also have an increased prevalence of nonthrombotic vascular occlusion. The aims of this work were to determine if aPLs directly promote medial hypertrophy or neointima formation in mice and to identify the underlying mechanisms.

Methods and Results—Medial hypertrophy and neointima formation invoked by carotid artery endothelial denudation were evaluated in mice administered normal human IgG or aPLs. While aPLs had no effect on medial hypertrophy, they caused exaggerated neointima development. This was related to an aPL-induced impairment in reendothelialization post denudation, and scratch assays in cell culture revealed that there are direct effects of aPLs on endothelium that retard cell migration. Further experiments showed that aPL antagonism of endothelial migration and repair is mediated by antibody recognition of β2-glycoprotein I, apolipoprotein E receptor 2, and a decline in bioavailable NO. Consistent with these mechanisms, the adverse impacts of aPLs on reendothelialization and neointima formation were fully prevented by the NO donor molsidomine.

Conclusions—APLs blunt endothelial repair, and there is related aPL-induced exaggeration in neointima formation after endothelial injury in mice. The initiating process entails NO deficiency mediated by β2-glycoprotein I recognition by aPLs and apolipoprotein E receptor 2. The modulation of endothelial apolipoprotein E receptor 2 function or NO bioavailability may represent new interventions to prevent the nonthrombotic vascular occlusion and resulting cardiovascular disorders that afflict antiphospholipid syndrome patients. (J Am Heart Assoc. 2014;3:e001369 doi: 10.1161/JAHA.114.001369)

Key Words: antiphospholipid syndrome • endothelium • neointima hyperplasia • nitric oxide

The antiphospholipid syndrome (APS) is an autoimmune disorder characterized by increased risk of thrombosis and cardiovascular disease resulting from elevated levels of circulating antiphospholipid antibodies (aPLs). The cardiovascular disorders for which APS patients have greater predisposition include coronary artery disease, myocardial infarction, and stroke. In addition to the thrombotic diathesis, the processes contributing to greater cardiovascular disease risk in APS include an increased prevalence of nonthrombotic vascular occlusion. APS patients display greater carotid artery intima-media thickness (IMT) and greater stenosis of the renal, intracranial, celiac, and mesenteric arteries than do non-APS subjects. Why APS patients have increased nonthrombotic vascular occlusion is unknown. In particular, whether aPLs directly promote processes that lead to nonthrombotic vascular occlusion and the potential mechanisms underlying such effects of aPLs on the vascular wall are unknown.

Numerous studies in cell culture and in animal models have revealed that pathologically relevant aPLs are directed against the phospholipid-binding protein β2 glycoprotein I (β2GPI). β2GPI is a plasma protein composed of 5 distinctive domains (domains I to V), and antibodies against domain I are an important disease-promoting component of aPLs. We previously demonstrated in cultured endothelial...
cells and in mice that aPL binding to domain I of β2GPI and the resulting interaction of β2GPI with the low-density lipoprotein receptor family member apolipoprotein E receptor 2 (ApoER2) in endothelium attenuate endothelial NO synthase (eNOS) activation, thereby promoting leukocyte–endothelial cell adhesion and thrombosis.21

In the current work, we designed experiments to test the hypothesis that aPLs promote medial hypertrophy and/or neointima formation. We used a mouse model that entails the induction of both of these processes after endothelial denudation in the carotid artery. The selection of such a model is based on recognition that an impairment in endothelial cell monolayer integrity most often underlies the initiation of medial hypertrophy or neointima formation.22–26 The results obtained reveal for the first time that although the development of medial hypertrophy is unaffected by aPLs, aPLs cause exaggerated neointima formation. Additional studies showed that this is related to a marked blunting of endothelial repair by aPLs involving direct actions on endothelial cells and that the impairment in reparative capacity is mediated by aPL recognition of domain I of β2GPI and ApoER2 and a decline in bioavailable NO. In contrast, aPLs have no direct effect on vascular smooth muscle cell (VSMC) proliferation or migration. We further demonstrated that aPL-induced attenuation of reendothelialization and the resulting exaggeration of neointima formation in vivo are fully prevented by the NO donor molsidomine. Recognizing that NO is also antithrombotic,27–29 strategies to enhance bioavailable NO abundance or action may provide new mechanism-based means to combat both the thrombotic and the nonthrombotic vascular complications of APS.

Materials and Methods

Animal Models

In vivo studies were performed in male C57BL/6 mice, in male ApoER2+/- and ApoER2-/- littermates on an identical 129SvEvxC57BL/6J background, and in male FVB mice.21,30 All animal experiments were approved by the Institutional Animal Care and Utilization Committees at the University of Texas Southwestern Medical Center and the University of Cincinnati.

Table. Clinical and Laboratory Features of the Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>aCL (≥140 GPLU)</th>
<th>Anti-β2GPI</th>
<th>Clinical Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>Arterial thrombosis, pregnancy losses, catastrophic APS, myocardial infarction</td>
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<tr>
<td>2</td>
<td>57</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>Arterial thrombosis, recurrent pulmonary hemorrhage, catastrophic APS</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>Adrenal hemorrhage, skin necrosis, hemolytic anemia, multiple pulmonary emboli, catastrophic APS</td>
</tr>
</tbody>
</table>

aCL indicates anti-cardiolipin antibodies; GPLU, IgG phospholipid units; β2GPI, β2-glycoprotein I; APS, antiphospholipid antibody.

Antibody Preparation

Polyclonal aPLs were isolated from patients with APS, which were characterized as having high-titer aPL levels (>140 IgG phospholipid units GPL U), thromboses, and/or pregnancy losses.21 The relevant clinical and laboratory features of the patients who provided aPLs are given in Table. Individuals provided informed consent before participating in these studies, and all protocols were approved by the institutional review boards of the Hospital for Special Surgery and the University of Texas Southwestern Medical Center. Human IgG from healthy nonautoimmune individuals (NHIgG) was purified by using an identical method. All IgG samples were treated to deplete endotoxin as previously described.21 Mouse monoclonal antibodies directed to domain I of β2GPI (3F8) and the control mouse monoclonal antibody (BBG) were prepared as previously reported.21,31

Carotid Artery Reendothelialization

Carotid artery reendothelialization was evaluated after perivascular electric injury in 12- to 16-week-old male mice as previously described.32,33 Mice were anesthetized via intraperitoneal administration of avertin, and the left common carotid artery was exposed through an anterior incision of the neck. Electric current of 4 W was applied through 2-mm forceps (2 W/mm) for 3 sec in microbipolar mode (Force 2 Electrosurgical Generator; Valleylab). The mice were allowed to recover, and analgesia was provided via intraperitoneal administration of buprenorphine immediately after surgery and 24 hours later. At 72 hours after injury, animals were injected intravenously with 5% Evans blue dye (Sigma-Aldrich), the carotid arteries were harvested, and the area of denudation (which incorporates the dye) was quantified in a blinded manner through image analysis by using Scion Image (free software from National Institutes of Health). NHIgG or aPLs (100 µg per mouse) were injected intraperitoneally 24 hours before injury, on the day of injury, and 24 hours after injury. For evaluation of the effect of molsidomine on reendothelialization, mice were injected intraperitoneally with molsidomine (Sigma-Aldrich, 5 mg/kg per day) or vehicle (saline) daily starting 72 hours before injury and ending 48 hours after injury.
Carotid Artery Neointima Formation

The procedures used to induce endothelial denudation in mouse carotid arteries, tissue preparation, and the evaluation of neointima formation were performed as described previously.30,34 Studies were performed in 8-week-old male FVB mice injected intraperitoneally with NHIgG (100 μg/mouse) or aPLs (100 μg/mouse) 24 hours before and every 48 hours after the endothelial injury for 14 days. Briefly, to denude the carotid artery endothelium, mice were anesthetized with a mixture of ketamine (80 mg/kg; Fort Dodge Laboratories) and xylazine (16 mg/kg; The Butler Co.). The left external carotid artery was looped proximally and ligated distally with 7-0 silk sutures (Ethicon). An epon-resin probe was produced by forming a bead slightly larger than the diameter of the carotid artery (~0.45 mm) on a 3-0 nylon suture. A transverse arteriotomy was made between the 7-0 sutures, the resin probe was inserted, and the probe was advanced toward the aortic arch and withdrawn 5 times. The probe was then removed and the proximal 7-0 suture was ligated. Mice were killed 1 hour or 14 days after injury by perfusion fixation with 10% phosphate-buffered formalin (pH 7.0) for 15 min at physiological pressure. The entire block of neck tissue containing both uninjured right and injured left common carotid arteries was harvested, fixed with the same solution for an additional 48 hours, and decalcified before being embedded in paraffin. Ten identical 5-μm cross sections at 500-μm intervals were made from the distal aspect of the neck beginning at the location of the proximally ligated 7-0 suture. Parallel sections were subjected to routine hematoxylin–eosin staining and to Verhoeff–Van Gieson staining of elastic lamina. For each animal, 4 whole-neck cross sections were digitized, and the vessels were quantified with Scion Image analysis software (Scion). Measurements of luminal area, area inside the internal elastic lamina, and the area encircled by external elastic lamina were made on each section. Medial area was calculated by subtracting internal elastic lamina from external elastic lamina area, and intimal area was calculated by subtracting luminal area from internal elastic lamina. To evaluate the effect of molsidomine on neointima formation, mice were injected intraperitoneally with molsidomine (5 mg/kg per day) or saline vehicle daily from 1 to 4 weeks.

Endothelial Cell Proliferation and Migration

For the evaluation of endothelial cell proliferation, BAECs were seeded in 6-well plates at 1 × 10^5 cells/well and grown in EGM2 at 37°C for 24 hours. Cells were then treated with NHIgG (100 μg/mL) or aPLs (100 μg/mL), and cell numbers were counted at 0, 24, and 48 hours. To study endothelial cell migration, BAECs were grown to near-confluence in 6-well plates and placed in 1% lipoprotein-deficient serum (provided by Dr J. Goldstein and Dr M. Brown, University of Texas Southwestern Medical Center) in DMEM for 16 hours, and a defined region of cells was removed by using a single-edged razor blade.32,33 Cells were treated with vascular endothelial growth factor (VEGF) (50 ng/mL) in the presence or absence of NHIgG or aPLs (100 μg/mL), control IgG (BBG), or β2GPI monoclonal antibody 3F8 (0.3 μg/mL) in DMEM+1% lipoprotein-deficient serum. In select studies, the cells were also incubated in the absence versus presence of S-nitroso-N-acetyl-dl-penicillamine (SNAP, 20 μmol/L) or hydroxyurea (1 mmol/L).35 Twenty-four hours later, the cells were fixed with 3% paraformaldehyde (Sigma-Aldrich), permeabilized in 0.2% Triton X-100 (Bio-Rad Laboratories), stained with hematoxylin (Fisher Scientific), and viewed under an inverted microscope (Zeiss Axiosvert 100 M). The number of cells migrated past a 2000-μm length of the wound edge was quantified in a minimum of 3 × 40 magnification) fields per well. All endothelial cell culture findings were confirmed in 3 independent experiments.

Cell Culture and RNA Interference

Primary bovine aortic endothelial cells (BAECs) were isolated by using previously described methods,21,33 cultured in EGM2 media (Lonza), and used within 3 to 7 passages. In small interfering RNA (siRNA) experiments, BAECs were transfected with siRNAs by using LipofectAMINE 2000 (Invitrogen) and studied 48 hours later.21,33 dsRNA with sequence 5’-ACUGGAAGCGGAAGAAUAC-3’ designed to target the open reading frame of bovine ApoER2 (accession number XM_865091) and control dsRNA were purchased from Dharmacon.21 Immunoblot analyses were performed to evaluate effective knockdown of ApoER2 expression.

Primary aortic VSMCs were isolated from C57BL/6 mice by using previously reported procedures with some modifications.30 Briefly, thoracic aortas were dissected from the mice, adventitial tissue was removed, and the aortas were incubated in Hanks’ solution containing 1 mg/mL collagenase and 0.5 mg/mL elastase for 30 min at 37°C. The resulting cell suspension was centrifuged at 150g for 5 min and resuspended in 10 mL of DMEM containing 10% FBS. The identity of the isolated cells as VSMCs was confirmed by positive immunostaining with anti-α-SMA monoclonal antibody (Sigma; clone 1A4). Cells were maintained in DMEM (Sigma-Aldrich) containing 10% FBS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin, and experiments were done at passages 1 to 4.

VSMC Proliferation and Migration

VSMC proliferation was assessed by using previously described methods.36 Briefly, cells were seeded in 96-well
culture plates at 1×10^4 cells/well and cultured in DMEM containing 0.2% FBS for 48 hours. The cells were incubated with or without PDGF-BB (30 nmol/L, Invitrogen) in the presence of NHlgG (100 µg/mL) or aPLs (100 µg/mL) for 18 hours at 37°C, followed by incubation with [3H]thymidine (0.2 µCi/well, 28 nmol/L; PerkinElmer) for 6 hours; washed with PBS; and lysed with 0.25 mol/L sodium hydroxide. Radioactivity in cell lysates was quantified with the use of liquid scintillation counting. VSMC migration was evaluated in a Transwell apparatus by using a membrane filter with pore size of 8.0 µm. After plating at 2×10^4 cells/well, cells were treated with or without PDGF-BB (30 nmol/L) for 4 hours. Nonmigrating cells on the upper surface were scraped gently and removed with PBS wash and wiping with a cotton-tipped applicator. The filters were fixed in ice-cold methanol and stained with DAPI, and the number of cells/HPF that had migrated into the membrane was counted microscopically (NIKON Eclipse TE2000-E, ×40 magnification, 5 fields/chamber). All VSMC culture findings were confirmed in 3 independent experiments.
Statistical Analysis

All data are expressed as mean±SEM. For cell culture experiments, Student t test or 1-way ANOVA was used to assess differences between 2 groups or among >2 groups, respectively, with Newman–Keuls post-hoc testing after ANOVA. Findings for the experiments performed in mice were evaluated by using Mann–Whitney tests to assess differences between 2 groups and Kruskal–Wallis analyses followed by Mann–Whitney with Bonferroni correction for comparisons of >2 groups. P<0.05 values were considered significant.

Results

Impact of aPLs on Medial Hypertrophy, Neointima Formation, and Endothelial Repair

To determine if aPLs promote medial hypertrophy and/or neointima formation, we assessed the impact of aPLs on these processes invoked by carotid artery endothelial denudation in mice. Using our previously established methods,30 the carotid arteries of male wild-type mice were denuded with an epon-resin intraarterial probe, and the mice were injected with NHlgG or aPLs every 48 hours for 14 days. At the end of the study period, whole neck sections were processed for histological analysis. Compared with noninjured carotid arteries, the endothelial denudation caused medial hypertrophy in both NHlgG- and aPL-treated mice, and aPLs did not enhance the hypertrophy (Figure 1A and 1B). However, endothelial denudation–induced neointima formation was markedly greater in mice injected with aPLs compared with mice given NHlgG (Figure 1A and 1C). Carotid artery lumen area was also reduced in aPL-treated versus NHlgG-treated mice (50 904±26 550 μm² versus 76 727±20 380 μm², respectively, mean±SEM, n=9 or 10, P<0.05). Thus, aPLs promote neointima formation.

Because neointima formation most often is initiated by a loss of endothelial cell monolayer integrity and the severity of neointima development is related to the capacity for endothelial cell monolayer repair,22–26 the effect of aPLs on carotid artery reendothelialization was then tested by using a thermal injury model in which the degree of reendothelialization is readily quantified. The mice received intraperitoneal injections of NHlgG or aPLs 24 hours before injury, on the day of injury, and 24 hours after injury. At 72 hours after injury, the area of remaining denudation was determined by intravascular injection of Evans blue dye and evaluation of its uptake by the carotid artery intimal surface. In a parallel set of studies in mice injected with Evans blue dye later on the day of injury, it was confirmed that the area of initial denudation was similar in mice injected with NHlgG versus those injected with aPLs (data not shown). Compared with mice administered NHlgG, mice treated with aPLs displayed a 6-fold larger area of remaining denudation at 72 hours after injury (Figure 2A and 2B). Thus, aPLs cause blunting of endothelial repair in vivo in mice.

Endothelial cells and platelets are major cellular targets of aPL action.17,37,38 To determine if there is a direct effect of aPLs on endothelial cells that alters their migratory capacity, scratch assays were performed by using cultured endothelial cells. After monolayer growth to near-confluence, endothelial cells were removed from a defined region, and cell migration

Figure 2. Antiphospholipid antibodies (aPLs) inhibit endothelial repair. A, Male wild-type mice received human IgG from healthy nonautoimmune individuals (NHlgG) or aPLs 24 hours before carotid artery endothelial denudation, on the day of denudation, and 24 hours after denudation. The remaining area of denudation was evaluated 72 hours later by assessing Evans blue dye uptake. Representative images of the carotid artery intimal surface displaying Evans blue dye incorporation are shown. B, Summary data for the area of remaining denudation is expressed in arbitrary units. Values are mean±SEM. For the NHlgG-treated group (n=6), median was 1610, and for the aPL-treated group (n=5), median was 10 710. *P<0.05 vs NHlgG. C, In scratch assays, bovine aortic endothelial cells (BAECs) were wounded and treated with media alone (nontreated [NT]), vascular endothelial growth factor (VEGF) (50 ng/mL), or VEGF in the presence of NHlgG (100 μg/mL) or 1 of 2 different isolates of aPLs (100 μg/mL) for 24 hours, and cell migration was evaluated. Representative images are shown. D, Summary data for migration. Values are mean±SEM, n=8/group, *P<0.05 vs NT, †P<0.05 vs NHlgG.
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The effect of NHIgG versus disruption to neointima development, aberrant VSMC growth (Figure 3B).

Inhibitory effect of antiphospholipid antibodies (aPLs) on scratch assay for endothelial cell migration does not involve effect on cell proliferation. A, Bovine aortic endothelial cells (BAECs) were seeded in 6-well plates (1 x 10⁵ cells/well) and grown for 24 hours. Cells were then incubated for 0, 24, and 48 hours in the presence of human IgG from healthy nonautoimmune individuals (NHIgG) (100 µg/mL) or aPLs (100 µg/mL), and the number of the cells/well was counted. There were no discernible effect on endothelial cell proliferation (Figure 3A), whereas BBG had no effect (Figure 5A).

In ApoER2 siRNA-decreased by 87% (control siRNA, 100.0±0.05). Representative immunoblots are shown in Figure 5B. The most pathologically relevant aPLs are directed against the phospholipid-binding protein β2GPI, and aPLs recognizing domain I of β2GPI in particular are highly associated with APS disease severity. To determine if β2GPI domain I recognition underlies aPL effects on endothelial cell migration, scratch assays were performed testing the effects of a monoclonal antibody to domain I of β2GPI (3F8) versus an IgG control (BBG). Mirroring the actions of polyclonal aPLs, 3F8 inhibited endothelial cell migration in response to VEGF, whereas BBG had no effect (Figure 5A).

The role of β2GPI and ApoER2

Because we and others previously determined that ApoER2 binds directly to the aPL–β2GPI complex adherent to endothelial cells, a potential requirement for ApoER2 in the antagonism of endothelial cell migration by β2GPI–recognizing antibody was demonstrated by siRNA knockdown of the receptor. In ApoER2 siRNA–treated cells, ApoER2 protein expression was decreased by 87% (control siRNA, 100.0±12.8% versus ApoER2 siRNA, 12.8±5.12%, n=6/group, mean±SEM, *P<0.05). Representative immunoblots are shown in Figure 5B. Although aPLs or anti-β2GPI monoclonal antibody 3F8 completely suppressed cell migration in control siRNA-transfected cells, the impact of aPLs or 3F8 was prevented in cells deficient in ApoER2 (Figure 5C and 5D).

To evaluate whether ApoER2 participates in the attenuation of endothelial repair by aPLs in vivo, carotid artery reend-
Figure 5. Endothelial cell repair inhibition by antiphospholipid antibodies (aPLs) requires β2-glycoprotein I (β2GPI) and apolipoprotein E receptor 2 (ApoER2). A, Scratch assays were performed with bovine aortic endothelial cells (BAECs) treated with media alone (basal), vascular endothelial growth factor (VEGF; 50 ng/mL), VEGF+isotype-matched control IgG (BBG, 0.3 μg/mL), or VEGF+3F8 monoclonal antibody to β2GPI (0.3 μg/mL). Values are mean±SEM, n=8/group, *P<0.05 vs basal, †P<0.05 vs BBG. B, BAECs were transfected with control silent interfering RNA (siRNA) or siRNA targeting ApoER2, and whole-cell lysates were immunoblotted for ApoER2 and actin. C, Scratch assays were performed 48 hours later with treatments with media alone (basal), VEGF (50 ng/mL), VEGF+human IgG from healthy nonautoimmune individuals (NHIgG; 100 μg/mL), or VEGF+aPLs (100 μg/mL) for 24 hours. Values are mean±SEM, n=4 for no treatment (ApoER2 siRNA) and VEGF, n=6 for no treatment (Control siRNA), VEGF+NHIgG, and VEGF+aPLs*P<0.05 vs basal, †P<0.05 vs NHIgG, #P<0.05 vs Control siRNA. D, Scratch assays were performed 48 hours after cell transfection with control siRNA or siRNA targeting ApoER2, using the treatments described for A. Values are mean±SEM, n=4 for no treatment and VEGF treatment group, and n=8 for VEGF+BBG and VEGF+3F8 treatment group, *P<0.05 vs basal, †P<0.05 vs BBG, #P<0.05 vs Control siRNA. E, Male ApoER2−/− or ApoER2−/− littermates treated with NHIgG (100 μg/mouse) or aPLs (100 μg/mouse) underwent carotid artery endothelial denudation, and the remaining area of denudation was evaluated 72 hours later. Representative images of the carotid artery intimal surface displaying Evans blue dye incorporation are shown. F, Summary data for the area of remaining denudation at 72 hours are expressed in arbitrary units. Values are mean±SEM. For ApoER2−/− n=4 (uninjured, median was 161.5) and n=5 (injured, median was 19 087). For ApoER2−/−, n=5 (uninjured, median was 7736) and n=5 (injured, median was 5923). *P<0.05 vs NHIgG in ApoER2−/−, †P<0.05 vs aPLs in ApoER2−/−.
othelialization experiments were performed in ApoER2+/+ versus ApoER2−/− mice administered NHIgG or aPLs. In NHIgG control–treated mice, reendothelialization was reduced in ApoER2−/− versus ApoER2+/+ mice (Figure 5E and 5F). This finding indicates that there is a role for the receptor in endothelial repair under basal conditions, and this was confirmed in nontreated ApoER2+/+ and ApoER2−/− mice (data not shown). Consistent with the observations in Figure 2A and 2B, aPL treatment of ApoER2+/+ mice caused the attenuation of reendothelialization. In contrast, aPL treatment did not further impair reendothelialization in ApoER2−/− mice (Figure 5E and 5F). These cumulative findings indicate that the recognition of domain I of β2GPI by aPLs and ApoER2 are critically involved in the adverse effects of aPLs on endothelial repair.

Role of NO Deficiency

Endothelial migration is governed by both NO-dependent and NO-independent processes. Having previously found that aPL actions via ApoER2 antagonize eNOS, we determined whether NO deficiency underlies the suppression of endothelial cell migration by aPLs (Figure 6A). Although aPLs inhibited migration stimulated by VEGF, the antagonistic effect of aPLs was fully prevented by the NO donor SNAP. To potentially link β2GPI recognition, NO deficiency, and the attenuation of endothelial cell migration, parallel experiments were performed with the anti-β2GPI monoclonal antibody (3F8) (Figure 6B). Mirroring the findings with aPLs, SNAP reversed the blunting of VEGF-stimulated migration by 3F8. To determine if exogenous NO can counteract the negative

Figure 6. Inhibition of endothelial cell migration and repair by antiphospholipid antibodies (aPLs) entail NO deficiency. A, Scratch assays were performed with bovine aortic endothelial cells (BAECs) treated with media alone (basal), vascular endothelial growth factor (VEGF; 50 ng/mL), VEGF+human IgG from healthy nonautoimmune individuals (NHIgG; 100 μg/mL), VEGF+aPLs (100 μg/mL), VEGF+NHIgG+S-nitroso-N-acetyl-0-l-penicillamine (SNAP, 20 μmol/L), or VEGF+aPLs+SNAP for 24 hours. Values are mean±SEM, n=8/group, *P<0.05 vs basal, †P<0.05 vs NHIgG, ‡P<0.05 vs no SNAP. B, Scratch assays were performed with BAEC as described in (A), using the control IgG BBG or anti-β2GPI antibody 3F8 instead of NHIgG or aPLs, respectively. Values are mean±SEM, n=8/group, *P<0.05 vs basal, †P<0.05 vs BBG, ‡P<0.05 vs no SNAP. C, Carotid artery reendothelialization was evaluated in aPL-treated male C57BL/6 mice administered vehicle (saline) or molsidomine (5 mg/kg per day). Representative images of the carotid artery intimal surface displaying Evans blue dye incorporation are shown. D, Summary data for the area of remaining denudation at 72 hours are expressed in arbitrary units. Values are mean±SEM, n=6 for saline-treated group (median was 7568) and n=7 for molsidomine-treated group (median was 182). *P<0.05 vs saline.
impact of aPLs on endothelial repair in vivo, the effect of the NO donor molsidomine was evaluated.\textsuperscript{45} APL-treated mice were concurrently administered either vehicle control (saline) or molsidomine (5 mg/kg per day), which produces the metabolite 3-morpholino-sydnonimine that generates NO.\textsuperscript{45} Paralleling the findings in cell culture with the NO donor SNAP, despite receiving aPLs, the remaining area of denudation 72 hours after injury was markedly reduced in the molsidomine-treated mice (Figure 6C and 6D). It was further tested whether the NO donor prevents aPL-induced neointima formation. Although molsidomine did not affect medial area (Figure 7A and 7B), aPL-induced neointima formation was reduced in mice given the NO donor (Figure 7A and 7C). The lumen area was also increased in molsidomine-treated mice (83 650±23 203 µm\(^2\), mean±SEM, n=8) compared with mice treated with saline (56 575±19 409 µm\(^2\), n=9, \(P<0.05\)). These findings reveal that NO deficiency underlies the adverse impacts of aPLs on endothelial monolayer repair and neointima formation.

**Discussion**

In addition to having greater risk for episodic occurrence of thrombosis, APS patients are predisposed to nonthrombotic vascular disorders. These include exaggerated carotid artery IMT, which is a preclinical indicator of atherosclerosis evident in APS patients independent of other cardiovascular risk factors,\textsuperscript{4–6,8,46,47} and arterial stenosis in various sites.\textsuperscript{9,11–15} In the present study, we demonstrate for the first time that aPLs promote neointimal hyperplasia after endothelial injury in mice. We further show that this is related to the attenuation of endothelial monolayer repair and that this involves direct adverse effects of aPLs on endothelial cells. When considered along with our prior discovery that eNOS antagonism by aPLs underlies the thrombotic diathesis characteristic of APS,\textsuperscript{21} these new observations indicate that direct actions of aPLs on endothelium likely play a major role in both the thrombotic and the nonthrombotic components of vascular disease in individuals with APS.

There are several clinical disorders in which the effects of aPLs on endothelial repair that adversely affect vascular smooth muscle behavior may be applicable. One such disorder is stroke, because APS patients with increased IMT have 3 times greater risk for stroke than do APS patients without IMT.\textsuperscript{8} A second condition is peripheral vascular disease, which is often a proliferative vasculopathy that leads to stenosis. Patients with APS are 80% more likely to undergo lower extremity vascular surgery than are patients without APS,\textsuperscript{48} and APS is an independent risk factor for more rapid progression of lower extremity occlusive vascular disease.\textsuperscript{49} APS patients are also more likely to have premature restenosis of coronary artery bypass grafts.\textsuperscript{50} Thus, the processes revealed in the current work are relevant not only to preclinical forms of vascular disease such as IMT but also to clinically apparent vascular disorders in APS patients.

Although originally thought to directly recognize anionic phospholipids on the surface of target cells, it has become apparent that pathologically relevant aPLs are directed against the phospholipid-binding protein \(\beta 2\text{GPI}\).\textsuperscript{17–19} \(\beta 2\text{GPI}\) is a plasma protein composed of 5 distinct domains (domains I to V), and antibodies against \(\beta 2\text{GPI}\) increase adhesion molecule expression and the synthesis of cytokines, endothelin-1, and tissue factor by cultured endothelial cells.\textsuperscript{20,51} Recent work has further revealed that antibodies specifically recognizing domain I of \(\beta 2\text{GPI}\) are an important pathogenetic component of aPLs.\textsuperscript{17–20} We previously showed that monoclonal antibodies to domain I of \(\beta 2\text{GPI}\) cause potent inhibition
of eNOS.21 eNOS plays an important role in endothelial repair and in protection against neointimal hyperplasia.43,52 Now we demonstrate in culture that a monoclonal antibody against β2GPI domain I inhibits endothelial cell migration, mimicking the effect of polyclonal human aPLs. In addition, we have implicated ApoER2 in aPL antagonism of cultured endothelial cell migration and in aPL attenuation of endothelial repair in vivo. We and others previously identified ApoER2 as a key transmembrane receptor operative in aPL effects on both endothelial cells and platelets.21,42,53 APL binding to β2GPI induces β2GPI dimerization, which allows the aPL–β2GPI complex to avidly bind to ApoER2.21,53,54 ApoER2 is required for aPL-induced antagonism of endothelial cell eNOS and the subsequent increase in leukocyte–endothelial cell interaction and thrombus formation.21,42 Using NO donors both in cell culture and in vivo, we now demonstrate that NO deficiency also participates in aPL antagonism of endothelial cell migration and repair. Intricate strategies such as peptide blockade of β2GPI interaction with ApoER2 have been contemplated to combat the clinical sequelae of APS.55–57 However, because we now know that eNOS antagonism underlies both APS-associated thrombosis21 and aPL-related defects in endothelial monolayer repair contributing to nonthrombotic vascular occlusion, more practical treatments aimed at enhancing NO bioavailability or action can now be considered to combat both the thrombotic and nonthrombotic components of APS-associated vascular disease.

In addition to resident endothelial cells in the arterial wall, circulating endothelial cells and endothelial progenitor cells may contribute to vascular repair in vivo.58,59 However, these cell populations are not altered in APS patients,60,61 and as such it is unlikely that changes in their function contribute to the attenuation in endothelial repair caused by aPLs in the current study. Leukocytes also modulate vascular repair62,63 and aPL antagonism of eNOS promotes leukocyte–endothelial adhesion,21 and the antibodies also induce tissue factor expression in monocytes,17,18 which can promote neointima formation.64 Thus, there may be processes besides the presently described direct impact of aPLs on endothelial cell migration that may additionally contribute to the exaggerated neointima hyperplasia observed in our study.

Along with experiencing greater arterial and venous thrombosis and increased risk of coronary artery disease, myocardial infarction, and cerebrovascular disease,3 female patients with APS have increased complications during pregnancy, including fetal loss, preterm birth, intrauterine growth restriction, preeclampsia, and eclampsia.1,17,65 Although the current work focuses on processes relevant to diseases unrelated to pregnancy, the mechanisms elucidated may also be applicable to the obstetric complications of APS because inhibitory effects of aPLs on primary human endometrial endothelial cell angiogenesis have been reported.66,67 In these studies, there was decreased tube formation in matrigel in culture and blunted in vivo angiogenesis in mice caused by aPLs, as well as aPL suppression of endothelial cell VEGF production. Now, knowing that the blunting of endothelial cell migration by aPLs entails ApoER2 and a decrease in NO bioavailability and that the motility of both systemically derived and endometrial endothelium are tempered by aPLs, the participation of ApoER2 and NO deficiency in preclinical models of obstetric APS can be tested.

Although there is considerable knowledge of the basis for APS-related thrombosis, how APS affects nonthrombotic vascular disease has been more enigmatic. We now report that aPLs promote neointima formation after endothelial injury in mice, that this is related to the blunting of endothelial repair, and that the attenuation of endothelial repair by aPLs entails NO deficiency mediated by β2GPI and ApoER2. The modulation of endothelial ApoER2 function or NO bioavailability may represent new interventions to prevent the nonthrombotic vascular occlusion and resulting cardiovascular disorders that afflict APS patients.

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References

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