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Inhibition of Nitric Oxide and Antiphospholipid Antibody-Mediated Thrombosis

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Abstract

The antiphospholipid syndrome (APS) is characterized by recurrent vascular thrombosis, thrombocytopenia and fetal loss occurring in the presence of antiphospholipid antibodies (aPL). Along with arterial and venous thrombosis and pregnancy complications, patients with APS have an increased risk of myocardial infarction, stroke and coronary artery disease, resulting from vascular cell dysfunction induced by aPL. Accumulating evidence to date indicates that interactions between circulating aPL and cell surface molecules of target cells, primarily endothelial cells and platelets, underlie the vascular disease phenotypes of APS. However, the molecular basis of APS is poorly understood. Nitric oxide produced by endothelial cells is a key determinant of vascular health that regulates several physiologic processes including thrombosis, endothelial-leukocyte interaction, vascular cell migration, and the modulation of vascular tone. This review will discuss recent findings that indicate a novel mechanism by which aPL antagonize endothelial cell production of nitric oxide and thereby promote thrombosis.

Keywords

Antiphospholipid syndrome; Apolipoprotein E receptor 2; β2-glycoprotein I; Endothelium; Endothelial nitric oxide synthase; Nitric oxide; Platelets; Thrombosis; Nitric oxide; Inhibition

Introduction

The antiphospholipid syndrome (APS) is a systemic autoimmune disorder marked by the presence of antiphospholipid antibodies (aPL) in the circulation that contribute to enhanced risk for vascular thrombosis and pregnancy complications[1-4]. APS afflicts a significant number of patients with systemic lupus erythematosus (SLE), with as many as 34% of SLE patients having circulating aPL, as well as individuals without another underlying disorder[5]. Patients with APS also have an increased risk of cardiovascular diseases, such as coronary artery disease, myocardial infarction, and stroke stemming from vascular cell dysfunction[6]. Nitric oxide (NO) is a key determinant of vascular health that regulates several physiologic processes including thrombosis, endothelial-leukocyte interaction, vascular cell migration and proliferation, and the modulation of vascular tone and permeability[7]. Impaired NO bioavailability represents a central feature of endothelial and platelet dysfunction that contributes to intravascular thrombosis and a number of vascular diseases. The primary source of NO in the vascular wall under normal conditions is the endothelial isoform of NO synthase (eNOS).

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This review will provide a brief overview of the role of NO and eNOS in regulation of platelet activation and thrombosis. It will then highlight the recent findings both in cultured cells and in mouse models that demonstrate the antagonism of eNOS by aPL. The molecular mechanisms by which aPL cause eNOS inhibition and thrombosis will be discussed in details. Novel interventions directly based on the pathogenetic mechanisms will be further considered that may be rapidly translated into new prophylactic or therapeutic strategies to combat the devastating impact of APS.

1. Anti-thrombotic Actions of Nitric Oxide

NO is a critical signal transduction molecule in the vascular system. NO is produced by three subtypes of NOS; nNOS (neuronal NOS, or NOSI), iNOS (inducible NOS or NOSII) and eNOS (endothelial NOS or NOSIII). The primary source of NO in the vascular wall under normal conditions is the endothelial isoform of NO synthase (eNOS). In addition to the endothelium, platelets and megakaryocytes express eNOS and they synthesize NO upon stimulation by a variety of agonists including thrombin and insulin [8-11].

The role of NO in the development of thrombosis has been investigated in animal models using inhibitors of NOS and its substrate L-arginine. In a rat model of thromboembolic stroke, infusion of the NOS inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME) caused an increase in platelet deposition and a reduction in global flow[12], indicating that both thrombotic and hemodynamic determinants contribute to the enhanced cerebral stroke. The role of endogenous NO production in the development of glomerular thrombosis associated with septic shock was studied an endotoxin-induced model of renal thrombosis[13]. Administration of endotoxin increased NO production, and this effect was inhibited by infusion of L-NAME. Kidneys from rats given endotoxin and L-NAME showed enhanced thrombosis in glomeruli as compared to those from rats given either endotoxin or L-NAME alone. In a rat model of nephrotoxic nephritis, enhanced NO production was observed, and the animals depleted of plasma L-arginine developed systemic hypertension and glomerular thrombosis, suggesting that the enhanced production of NO in this condition prevents acute glomerular injury[14]. In a canine model of coronary occlusion, thrombus formation in the coronary artery was delayed by administration of L-arginine[15]. Infusion of L-arginine also enhanced lysis of thrombus in the coronary arteries and inhibited platelet aggregations ex vivo. In a study of rabbit mesenteric venules, inhibition of NOS by N^{ω}-nitro-L-arginine increased the duration of embolization and the number of emboli[16]. In contrast, infusion of L-arginine prevented the increase in venous embolization. Importance of eNOS in prevention of platelet aggregation and thrombosis has been also demonstrated in eNOS knockout mice[17]. These mice show increased propensity to thrombosis, stroke and atherosclerosis[18-21], and platelets isolated from eNOS deficient mice display enhanced aggregation[8]. The effect of exogenous NO on thrombosis has also been evaluated. Incubation with NO donor, S-nitroso-N-acetylcysteine, inhibits the upregulation of platelet surface glycoproteins including P-selectin and the integrin glycoprotein IIb/IIIa complex [22]. In a canine model of coronary artery stenosis associated with thrombus-dependent reductions in coronary blood flow, NO donor S-nitroso-bovine serum albumin reduced the frequency of flow cycles[23]. These cumulative studies in animal models highlight the anti-thrombotic actions of eNOS and NO.

The molecular basis of NO actions on platelets has been mainly attributed to its stimulation of soluble guanylate cyclase to produce cyclic GMP (cGMP)[24-26]. The increased production of cGMP leads to the stimulation of cGMP-dependent protein kinase that results in a reduction in fibrinogen binding to glycoprotein IIb/IIIa and modulation of phospholipase A2- and C-mediated responses[25;26]. NO also suppresses the agonist-dependent increase in cytosolic free calcium in a cGMP-dependent manner in isolated platelets[27]. More recently, cGMP-independent regulation of platelet function has also

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been reported[28;29]. NO suppresses exocytosis of Weibel-Palade bodies, endothelial granules that mediate vascular inflammation and thrombosis, by regulating the activity of N-ethylmaleimide-sensitive factor (NSF). NSF is a key component of the exocytic machinery, and NO inhibits NSF-mediated disassembly of soluble NSF attachment protein receptor complexes by nitrosylating critical cysteine residues of the factor.

Insufficient production of endogenous NO is also associated with thrombosis in clinical disorders in humans[30;31]. It is well established that thrombosis is a common cause of myocardial infarction and unstable angina[32;33], and platelets from patients with these conditions are activated with increased surface expression of P-selectin and active glycoprotein IIb/IIIa[34]. These platelets isolated from these patients produced less NO compared to patients with stable coronary artery disease, and the elevation of surface expression of P-selectin and glycoprotein GPIIb/IIIa was reduced by treatment with NO donors, such as nitroglycerin or S-nitrosoglutathione[35]. These results suggest that impaired NO production may contribute to the development of acute coronary syndromes by altering platelet function and consequent thrombus formation. Patients with atrial fibrillation, a condition associated with increased intracardiac thrombosis and cerebral embolism, have decreased plasma levels of nitrite and nitrate as well as lower levels of platelet cGMP[36]. Thrombosis has also been associated with NO deficiency in non-cardiac clinical disorders. Women with preeclampsia, a disorder with hypertension and intrarenal thrombosis during pregnancy, had lower levels of urinary cyclic GMP compared to noneclamptic women[37]. Recently, the role of human eNOS polymorphisms in thrombosis has been examined. Several polymorphisms in eNOS gene have been reported, including the ecNOS4a/4b in intron 4, the E298D mutation in exon 7, and the T786C in the promoter region. The E298D polymorphism of the eNOS gene was associated with an increased risk of hypertension, myocardial infarction and stroke in patients homozygous for this variant[38-41]. Another eNOS polymorphism ecNOS4a has been associated with severe stenosis in arteries and a history of myocardial infarction in smokers[42]. The polymorphism in the promoter region was associated with lower levels of platelet-derived NO and increased release of superoxide[43]. Taken together, these data suggest that select eNOS variants may influence thrombotic propensity in humans.

In summary, NO produced by eNOS in endothelial cells and platelets modulates a number of vascular processes including thrombosis that are known to be altered in APS patients.

2. NO Antagonism in Thrombosis in Antiphospholipid Syndrome

(a) eNOS Antagonism in Antiphospholipid Syndrome—As discussed above, eNOS and NO modulate critical vascular processes that are known to be adversely affected in APS. A potential link between APS and changes in bioavailable NO has been reported in both mouse models and humans. In mouse models, the administration of polyclonal aPL isolated from human patients or monoclonal β2GPI antibodies reduce plasma concentrations of NO metabolites and they also attenuate Ach-induced relaxation in isolated aortic rings, which is an NO-dependent process[60;61]. In humans, plasma aPL levels are inversely correlated with urinary NO metabolite excretion, and APS patients have lower levels of plasma nitrites compared to control subjects[62;63]. As such, the evidence both in mouse models and in humans supports a direct role for impaired NO production in the pathogenesis of APS.

This possibility has been directly investigated recently in studies by Ramesh et al., in which impact of aPL was tested in cultured endothelial cells and mouse models[64;65]. Cultured aortic endothelial cells were treated with aPL or normal human IgG (NHIgG) isolated from APS patients or healthy individuals, respectively, and their effects on eNOS activation induced by vascular endothelial growth factor (VEGF) was evaluated. Whereas NHIgG did not show any effect on VEGF-induced eNOS activation, aPL completely inhibited the

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activation. They further demonstrated that aPL induced increase in monocytes adhesion to the cultured endothelial cells and that this effect of aPL was prevented by addition of exogenous NO donor, S-Nitroso-N-acetylpenicillamine. These results demonstrate that aPL antagonize eNOS, leading to diminished NO production that induces monocytes adhesion in cultured endothelial cells. The antagonism of eNOS was further evaluated in vivo using carotid vascular conductance responses to acetylcholine (Ach) in mice, which are the indicator of endothelium-derived NO-dependent vascular relaxation[66]. Ach-mediated increases in carotid artery vascular conductance were assessed before and following the intravenous injection of either NHIgG or aPL. The mice injected with NHIgG showed normal response to Ach; in contrast, the mice injected with aPL showed marked attenuation of the vasodilatory response. These findings both in cell culture and in mice reveal for the first time that aPL have an inhibitory effect on eNOS.

Considering that NO plays a critical role in preventing thrombus formation and leukocyte adhesion to endothelium[67], Ramesh et al. further tested whether the eNOS antagonism by aPL is responsible for thrombus formation and leukocyte-endothelial cell adhesion in the mesenteric microcirculation using intravital microscopy[64]. Wild-type or eNOS deficient ($eNOS^{-/-}$) mice were treated with either NHIgG or aPL, and 24 hours later endothelial cell-leukocyte adhesion and thrombosis induced by ferric chloride were studied. A marked increase in leukocyte adhesion and thrombus formation was observed in aPL-treated wild-type mice. In contrast, in $eNOS^{-/-}$ mice aPL did not induce increase in either leukocyte adhesion or thrombosis. These results provide the causal link between eNOS antagonism and the increase in leukocyte-endothelial cell adhesion and thrombus formation induced by aPL.

(b) Molecular Basis of eNOS Antagonism by Antiphospholipid Syndrome

Involvement of β2-glycoprotein I: Antibodies against phospholipid-binding proteins, particularly β2-glycoprotein I (β2GPI) are the major pathogenic antibodies in APS, and elevated levels of circulating anti-β2GPI antibodies are directly associated with both vascular and obstetric complications in APS patients[68;69]. β2GPI is a plasma protein composed of five distinctive domains (domains I-V), and it has been demonstrated that domain V of the protein binds to phospholipids on the surface of target cells such as platelets[69]. Studies using in vitro system have found that binding of antibody to phospholipid-bound β2GPI induces its dimerization and conformational change, which further increase its affinity for negatively-charged phospholipids on the cell surface[70;71]. In cultured endothelial cells, anti-β2GPI monoclonal antibodies have been shown to enhance adhesion molecule expression and the synthesis of cytokines, endothelin-1, and tissue factor, and in isolated platelets they increase the production of thromboxane B_2, adhesion to collagen and aggregation[44;71-73]. In an animal model of photochemically induced arterial thrombosis, monoclonal β2GPI antibodies promote thrombus formation[47]. In APS patients, a strong relationship has been reported between the circulating levels of anti-β2GPI domain I antibodies and clinical symptoms[74-76].

Role of β2GPI in aPL antagonism of eNOS was first assessed by loss-of-function experiments comparing the actions of aPL in the presence or absence of β2GPI on the endothelial cell surface[64;77;78]. When cells were deprived of β2GPI, aPL did not cause eNOS inhibition, indicating that β2GPI is required for the aPL action. The role of β2GPI was further confirmed by evaluating the actions of anti-β2GPI monoclonal antibodies[79;80]. Monoclonal anti-β2GPI antibodies that specifically recognize domain I caused attenuation of VEGF-induced eNOS activation, mimicking the effect of the aPL that are isolated from APS patients. In contrast, another monoclonal antibody that recognizes domain II of β2GPI had no inhibitory effect on eNOS activation. The requirement for the

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β2GPI dimerization was further tested using purified β2GPI dimers on eNOS activation[64]. Whereas monomeric β2GPI had no effect on eNOS activation, dimerized β2GPI completely inhibited eNOS. These cumulative findings in cultured endothelial cells reveal that aPL-induced dimerization of β2GPI is required for aPL antagonism of eNOS, and that aPL binding to domain I of β2GPI likely mediates the process, providing a potential basis for the human studies[74].

Requirement for apoER2: Apolipoprotein E receptor 2 (apoER2), also known as LRP8, is a transmembrane protein that plays an important role in neuronal development as a signal transduction receptor for the glycoprotein Reelin[81]. Previous works using purified proteins in vitro have demonstrated that dimerized β2GPI binds to multiple members of the LDL receptor family including apoER2[82;83]. In addition to its abundant expression in brain, splice variants of apoER2 (designated apoER2') have been detected in platelets and megakaryocytic cell lines[82-84]. Recent studies in isolated platelets have shown that platelet activation induced by dimerized β2GPI requires apoER2'[83].

Since aPL antagonism of eNOS is mediated by dimeric β2GPI and that apoER2 is expressed in endothelial cells, Ramesh et al. investigated the requirement for LDL receptor family protein, particularly apoER2, in aPL-induced eNOS antagonism[64]. In cultured endothelial cells, the LDL receptor family inhibitor, RAP (receptor-associated protein) [85] prevented aPL inhibition of eNOS, indicating that LDL receptor family protein is required for the aPL action. The involvement of apoER2 in particular has been evaluated using the specific knockdown of apoER2 protein by small interference RNA (siRNA). Knockdown of the receptor markedly attenuated the inhibitory effect of aPL on eNOS activation. It also prevented the enhancement of monocytes adhesion to the endothelium caused by anti-β2GPI monoclonal antibody. The role of β2GPI-apoER2 interaction in aPL antagonism of eNOS has been further investigated using a small peptide inhibitor. In previous studies in isolated platelets, a soluble peptide based on the sequence of the first LDL-binding domain of apoER2, designated sBD1, prevented the interaction of β2GPI with the receptor[83;84]. In cultured endothelial cells, sBD1 fully prevented the inhibition of eNOS by aPL, indicating that interaction between BD1 of the apoER2 and domain V of β2GPI is required for aPL antagonism of eNOS. These findings in cultured endothelial cells have revealed that apoER2 plays a critical link that connects the aPL recognition of β2GPI on the endothelial cell surface with the intracellular events leading to eNOS antagonism.

Ramesh and the colleagues further evaluated the role of apoER2 in aPL-induced thrombus formation and leukocyte adhesion in vivo using apoER2$^{+/+}$ and apoER2$^{-/-}$ mice[64]. First to determine whether apoER2 is the transmembrane protein coupling aPL to eNOS, the effect of aPL on Ach-mediated increases in carotid vascular conductance was assessed in apoER2$^{+/+}$ and apoER2$^{-/-}$ mice. Whereas the administration of aPL caused marked reduction of the vasodilatory response to Ach in apoER2$^{+/+}$ mice, Ach-induced vasodilation was identical before and after aPL treatment in apoER2$^{-/-}$ mice. The involvement of apoER2 in aPL-induced increases in leukocyte adhesion to endothelium and in thrombus formation was also determined. ApoER2$^{+/+}$ or apoER2$^{-/-}$ mice were treated with NHIgG or aPL, and 24 hours later endothelial cell-leukocyte adhesion or thrombus formation was evaluated using intravital microscopy. In wild-type apoER2$^{+/+}$mice, aPL increased leukocyte adhesion to endothelium and it also enhanced thrombus formation. In contrast, aPL had no effect on leukocyte-endothelial cell adhesion or thrombosis in apoER2$^{-/-}$ mice. Importantly, the involvement of apoER2 in aPL-induced thrombosis has been independently demonstrated by other investigators[86]. They found that in wild-type mice human polyclonal aPL or murine anti-β2GPI monoclonal antibody increased tissue factor production and thrombus formation. In contrast, these pathologic effects of the antibodies were reduced in apoER2$^{-/-}$ mice. Furthermore, they showed that the blocking peptide sBD1

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that interferes with β2GPI-apoER2 interaction attenuates these pathologic events invoked by aPL or anti-β2GPI antibody. These recent cumulative findings indicate that apoER2 mediates aPL-induced eNOS antagonism and tissue factor upregulation, contributing to the development of the vascular disease phenotypes of APS.

Intracellular Signaling Pathway Involved in aPL antagonism of eNOS: The molecular basis for aPL antagonism of eNOS was further investigated using cultured endothelial cells focusing on the regulation of the critical phosphorylation of the enzyme[64]. eNOS stimulation by multiple agonists including VEGF entails phosphorylation at the critical serine residue of the enzyme (S1177 in human) by the upstream kinase Akt [87;88]. Pre-incubation with aPL, but not with NHIgG, caused inhibition of VEGF-induced S1177 phosphorylation. To determine the basis for impaired S1177 phosphorylation by aPL, potential changes in Akt activation were then assessed. Whereas aPL treatment blunted eNOS S1177 phosphorylation induced by VEGF, activation of upstream kinase Akt was unaffected. This result suggests that aPL attenuate eNOS phosphorylation of S1177 via a mechanism distal to Akt. The phosphorylation of the enzymatic is known to be negatively regulated by the protein phosphatase 2A (PP2A)[89;90]. The role of PP2A in aPL-induced changes in eNOS activity was evaluated by siRNA knockdown. Decreased expression of PP2A by RNAi prevented aPL inhibition of eNOS. These findings in cultured endothelial cells indicate that aPL attenuate eNOS phosphorylation and activation through activation of PP2A. The pathway in which aPL leads to eNOS antagonism is depicted in Figure 1.

Unanswered Questions

In addition to recurrent arterial and venous thrombosis, patients with APS suffer from increased risk of cardiovascular diseases. Recent studies shed new lights on the underlying molecular mechanisms of vascular phenotypes of the disorder, revealing that aPL antagonize eNOS through binding to β2GPI on the cell surface and its interaction with the plasma membrane receptor apoER2. Experiments in mouse models indicate that the inhibition of eNOS and resulting attenuation in bioavailable NO contributes to thrombus formation and leukocyte adhesion to vascular endothelial cells. However, there are many aspects of the molecular mechanisms of aPL action that warrant further investigation. First, we do not know how the membrane receptor apoER2 transduces the signal initiated by binding to aPL-βGPI. Possible roles for known adaptor molecules of apoER2, such as Disabled-1, need to be considered [81;83]. Second, it is entirely unknown how apoER2 activates the phosphatase PP2A. PP2A is a serine/threonine phosphatase which regulates many cellular functions[91]. PP2A is composed of structural scaffolding (A), catalytic (C) and regulatory (B) subunits. The activity and substrate specificity of PP2A can be regulated through post-translational modification of the catalytic C subunit and through regulation of the B subunits, which are responsible for targeting different phosphoprotein substrates to PP2A. Each B-subunit has a distinct tissue and/or subcellular localization. The ability of aPL to promote dephosphorylation of eNOS via PP2A is not unique to aPL; prior studies have shown that endostatin, endothelial differentiation-related factor-1, peroxynitrite, and C-reactive protein all inhibit eNOS activity through PP2A[66;92-94]. How any of these factors regulates PP2A activity on eNOS in endothelial cells is yet to be determined. Third, it is not known whether aPL exert similar inhibitory effects on eNOS in platelets, which express the splice variant of apoER2. Platelets also express eNOS, which has been shown to attenuate platelet activation in an autocrine manner [8;9]. Lastly, we do not know how the mechanism leading to eNOS antagonism by aPL would explain the episodic characteristics of the thrombotic diathesis in APS. Despite the persistent presence of aPL in circulation, APS patients most often do not show clinical symptoms, suggesting that the presence of aPL is necessary but not sufficient for thrombosis or pregnancy complications. A hypothesis called "two-hit hypothesis" has been suggested, in which additional stimuli are required for

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manifestation of the clinical symptoms[95]. It has been proposed that aPL may decrease the threshold for activation in endothelial cells and platelets (first hit), and clinical events occur after another triggering event (second hit). Does impaired NO production and subsequent endothelial dysfunction induced by aPL represent “the first hit”? If so, what is the “second hit” that triggers the thrombotic events in APS? It may involve a proinflammatory stimulus or infection because rats administered aPL have spontaneous thromboses if they also receive lipopolysaccharide[96]. We also do not know whether or how the “first hit” and the “second hit” are mechanistically linked. As the molecular basis that underlies the “first hit” is further investigated, the nature of the processes that constitute “second hit” directly leading to thrombosis will become clear.

Conclusions

Patients with APS are currently treated with chronic anticoagulation medications such as heparin and warfarin or with medications that modulate the immune response. Despite long-term use of the anticoagulants, recurrent clinical events are reported in APS patients. Furthermore, use of oral anticoagulants is associated with a high risk for multiple complications including bleeding episodes that require frequent monitoring[97-100]. Building upon the novel findings discussed in this review, we anticipate that novel interventions can be developed that directly target the pathogenetic mechanisms, thereby affording greater efficacy and fewer complications in the management of this potentially life-threatening disorder APS.

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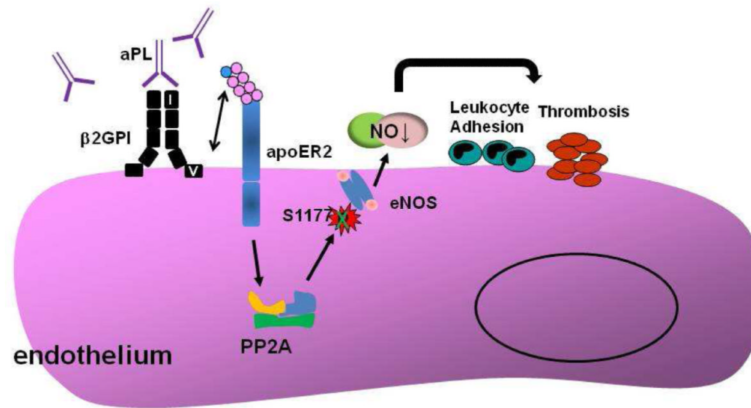

Figure 1.

The molecular mechanism of aPL antagonism of eNOS. Circulating aPL binding to domain I of β2GPI induces β2GPI dimerization, and interaction between domain V of β2GPI and the first LDL binding domain of apoER2 (shown in blue circle). Through yet-to-be-determined mechanism(s), the interaction of β2GPI with apoER2 causes increased activation of the phosphatase PP2A. This promotes the dephosphorylation of Ser1177 of eNOS yielding decreased enzyme activity and a decline in bioavailable NO, which contributes to increased leukocyte adhesion and enhanced thrombosis.