DISSECTING THE MOLECULAR BASIS OF THE
ANTIPHOSPHOLIPID SYNDROME

APPROVED BY SUPERVISORY COMMITTEE

Philip W. Shaul, M.D. (Mentor) _____

Shmuel Muallem, Ph.D. (Committee Chair) _____

David Karp, M.D., Ph.D. (Committee Member) _____

James Stull, Ph.D. (Committee Member) _____

To my family for their support and encouragement

DISSECTING THE MOLECULAR BASIS OF THE ANTIPHOSPHOLIPID SYNDROME

by

SANGEETHA RAMESH

DISSERTATION

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Sangeetha Ramesh, Ph.D.

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Supervising Professor: Philip W. Shaul, M.D.

The antiphospholipid syndrome (APS) is an autoimmune disorder characterized by circulating antiphospholipid antibodies (aPL), thrombotic events, recurrent pregnancy loss, and increased risk of coronary artery disease. The endothelium is a critical direct target of aPL, which cause increases in adhesion molecule expression and procoagulant activity. However, the molecular mechanisms underlying aPL actions on endothelium are unknown. Nitric oxide (NO) produced by endothelial NO synthase (eNOS) prevents leukocyte adhesion and thrombosis. In the present study we determined if aPL-induced alterations in

endothelial cell phenotype are mediated by aPL actions on eNOS. Normal human IgG (NHIgG) and human IgG containing polyclonal aPL were obtained from healthy individuals and APS patients, respectively, and purified by protein G-sepharose chromatography. We found that aPL prevents VEGF-mediated attenuation of monocyte adhesion to cultured endothelial cells and this is reversed by an NO donor, indicating a role for eNOS antagonism. In contrast, NHIgG has no effect on adhesion. Whereas NHIgG does not alter eNOS activation, stimulation of eNOS by VEGF and other agonists is fully antagonized by aPL. In intact mice, NO-dependent, acetylcholine-induced increases in carotid vascular conductance are unchanged by NHIgG treatment but impaired following aPL, indicating that these processes are operative *in vivo*. Additional studies in cultured endothelial cells demonstrated that aPL attenuates eNOS activation by inhibiting Ser1179 phosphorylation. We further found that deprivation of the cell surface protein β2 glycoprotein I (β2GPI) from endothelial cells prevents aPL inhibition of eNOS, and that FC1, a monoclonal antibody against β2GPI, mimics the effects of aPL. Receptor-associated protein or RAP, an antagonist of the LDL receptor (LDLR) family, fully prevents aPL antagonism of eNOS in endothelial cells. Moreover, eNOS antagonism by aPL as indicated *in vivo* in carotid vascular conductance studies is absent in apoER2 $^{-/-}$ mice. Thus, aPL-induced changes in endothelial cell phenotype are mediated by eNOS antagonism, which is due to impaired Ser1179 phosphorylation and requires β2GPI and apoER2. eNOS

antagonism through these mechanisms represent a novel, potentially critical proximal process in the pathogenesis of thrombosis and cardiovascular disease in APS.

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List of abbreviations

Ach	Acetylcholine
aCL	Anticardiolipin antibody
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AP	Activator protein
aPL	Antiphospholipid antibody
apoER2	Apolipoprotein E receptor 2
APP	Amyloid precursor protein
APS	Antiphospholipid Syndrome
Arg	Arginine
β2GPI	beta-2 glycoprotein I
BAEC	Bovine aortic endothelial cells
bFGF	Basic fibroblast growth factor
BH4	(6R)-5,6,7,8-tetrahydrobiopterin
BMP-4	bone morphogenic protein-4
BP	blood pressure
CaM/ CM	Calmodulin
CAPS	Catastrophic Antiphospholipid Syndrome
cGMP	cyclic guanine monophosphate

CRP	C-reactive protein
Crry-Ig	C3 convertase inhibitor
DMEM	Dulbecco's modified Eagle's medium
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
F(ab')2	Bivalent Fab fragment of antibody
FAD	Flavin Adenine Dinucleotide
FBS	Fetal bovine serum
FcRIIB	Fc receptor II B
Fe	Iron
FMN	Falvin Mononucleotide
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HAEC	Human Aortic Endothelial Cells
HDL	high density lipoprotein
Hsp	Heat shock protein
ICAM	Intracellular adhesion molecule
IL-1β	Interleukin-1 beta

IL-6	Interleukin-6
IMT	Intima-medial thickness
iNOS	inducible nitric oxide synthase
IUGR	Intrauterine growth retardation
kDa	Kilo dalton
LA	Lupus Anticoagulant
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
L-NAME	Nω-Nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
LRP	LDL receptor related protein
LysoPC	Lysophosphatidyl choline
MAC	Membrane attack complex
MAEC	Mouse Aortic Endothelial Cells
MAPK	p44/42 mitogen activated protein kinase
MAZ	myc-associated zinc finger protein
MCP-1	Macrophage chemoattractant protein-1
MFLM91U	mouse endothelial cells from embryonic lung mesenchyme
mRNA	messenger RNA
MvEC	Microvessel endothelial cells
MyD88	Myeloid differentiation primary response gene 88

Myr	Myristoylated
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF	nuclear factor
NHIgG	Normal human IgG
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOSIP	eNOS interacting protein
OxLDL	Oxidized low density lipoprotein
p38MAPK	p38 mitogen activated protein kinase
Palm	palmitoylated
PBS	Phosphate buffered saline
PDGF	platelet derived growth factor
PI3K	phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKG	Protein kinase G
PL	Phospholipid
PP	Protein phosphatase
RAP	Receptor associated protein
RNA	ribonucleic acid

SLE	Systemic Lupus Erythematosus
SNAP	S-nitroso-N acetyl-D, penicillamine
SPR	Surface plasma resonance
TGF-β	transforming growth factor beta
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
t-PA	tissue plasminogen activator
TRAP	Thrombin receptor agonist peptide
TxA2	Thromboxin A2
VCAM	Vascular cellular adhesion molecule
VEGF	Vascular endothelial growth factor
VLDL	very low density lipoprotein
VLDLR	Very low density lipoprotein receptor

Chapter 1

Introduction

Antiphospholipid Syndrome (APS)

The antiphospholipid syndrome (APS), also known as the Hughes Syndrome, is an autoimmune condition in which vascular thrombosis and/ or recurrent pregnancy losses occur in patients with circulating antiphospholipid antibodies (aPL) that recognize phospholipids or phospholipids binding cofactors. The disorder is classified as primary when it occurs in the absence of systemic lupus erythematosus (SLE) and secondary when SLE is present (Rand, 2003). Manifestations of APS are broad and include in addition to thrombosis and pregnancy morbidity, thrombocytopenia, livedo reticularis, cardiac valvular disease and an acute syndrome of multiorgan thrombosis termed as catastrophic antiphospholipid syndrome (CAPS).

Specific criteria known as the Sapporo Investigational Criteria have been developed to define APS (Rand, 2003). These include (1) clinical history of one or more events of vascular thrombosis (involving any site) or history of fetal loss and (2) laboratory evidence of a positive lupus anticoagulant (LA) test or a medium or high titer of anticardiolipin (aCL) IgG and/or IgM antibodies measured by an assay for beta 2 glycoprotein I (β2GPI)-dependent aCL

antibodies. The laboratory abnormalities should be present on two or more occasions at least six weeks apart. In the functional LA assay, aPL prolong phospholipids-dependent coagulation steps *in vitro* by competing with coagulation factors for binding to the phospholipid (Sammaritano, 2005). In the more sensitive but less specific aCL assay, aCL are detected by enzyme linked immunosorbent assay (ELISA) that utilizes cardiolipin as an antigen in the presence of β2GPI (Sammaritano, 2005). Increasingly, anti-β2GPI antibodies, detected by ELISA on β2GPI coated plates, have been used clinically though it requires further standardization and validation (Sammaritano, 2005).

It is not yet clearly understood how antiphospholipid antibodies (aPL) arise in patients with APS. Many infections are associated with increase in aPL (Shoenfeld et al., 2006). Bacterial and viral infections may be a trigger for the development of aPL in autoimmune diseases particularly in susceptible or genetically predisposed individuals (Gharavi et al., 2003). Pathogenic aPL may be generated in APS by molecular mimicry of cellular molecules including β2GPI that is a major cause of the thrombotic symptoms in APS (Gharavi et al., 1999; Gharavi et al., 2003; Shoenfeld et al., 2006). Thrombosis is presumed to cause many of the pregnancy complications associated with APS. The association between APS and fetal loss is strongest for loss occurring after 10 weeks. However, the association between aPL and the risk of premature birth due to eclampsia, preeclampsia and intrauterine growth retardation (IUGR) remains

controversial (Lim et al., 2006). Venous thromboembolism is the most common initial clinical manifestation of APS (Lim et al., 2006). Arterial thromboembolism occurs in APS patients most commonly in the cerebral circulation, and stroke (13%) and transient ischemic attacks (7%) are the initial clinical manifestations (Lim et al., 2006).

The current clinical management of patients with APS and venous thrombosis is a lifelong treatment regimen of anticoagulants including warfarin, unfractionated or low molecular weight heparin. Warfarin and aspirin are used for the prevention of thromboembolic complications in patients with a first ischemic stroke and aPL (Lim et al., 2006). For APS and pregnancy loss, combination therapy with unfractionated or low molecular weight heparin and aspirin significantly reduces pregnancy loss (Lim et al., 2006).

Mechanisms of Fetal Loss in APS

Up to one quarter of women with recurrent pregnancy loss have APS (Salmon et al., 2007). Before heparin therapy was recommended for management of pregnant patients with APS, the fetal loss rate was more than 50%. Currently, after heparin therapy the fetal loss rate is less then 20% (Salmon et al., 2007).

aPL cause a prothrombotic phenotype by causing dysfunction of endothelial cells, monocytes and platelets (described in detail later). aPL have also been shown to induce thrombosis in the placenta and placental vasculature by

impairing the annexin A5 anticoagulant shield on phospholipids surfaces of trophoblasts and attenuating intrinsic and extrinsic fibrinolysis (Atsumi et al., 2005; Rand and Wu, 2004). aPL also reduce the maturation and invasiveness of trophoblast cells *in vitro*, suggesting that aPL cause defective placentation in addition to causing increased thrombosis (Di Simone et al., 2000; Salmon et al., 2007).

Though the specific antigenic reactivity of aPL is crucial for their effect, the pathogenic mechanisms that lead to fetal and placental injury *in vivo* are not completely understood. Though antigens recognized by aPL are important, alteration of target antigen function is not sufficient for recurrent fetal loss (Salmon et al., 2003). In addition to thrombosis in placental tissue and the upregulation of proinflammatory factors, aPL also trigger the activation of endothelial cells, monocytes and platelets leading to tissue damage (Salmon et al., 2003). Complement activation is also a necessary *in vivo* step required for pathogenic effect of aPL on fetal well-being (Holers et al., 2002). These *in vivo* pathogenic effects require both the recognition of target antigens by aPL and Fc domain-mediated complement activation (Girardi et al., 2003; Salmon et al., 2003).

A murine model of APS has been developed in the laboratory of Dr. Jane E Salmon at the Hospital for Special Surgery and the Weill Medical College of Cornell University. In this model pregnant mice are injected with IgG isolated

from normal individuals (NHIgG) or from individuals with a high titer of aPL antibodies (aPL). The mice are injected on day 8 and 12 of pregnancy and the uteri are harvested on day 15. In mice injected with aPL the amnionic sacs are small, approximately 40% of the embryos are resorbed and the ones that survive are growth restricted (**Figure 1-1A**) compared to mice injected with NHIgG where the amnionic sacs are of normal size and there is no fetal resorption (**Figure 1-1B**) (Girardi et al., 2003).

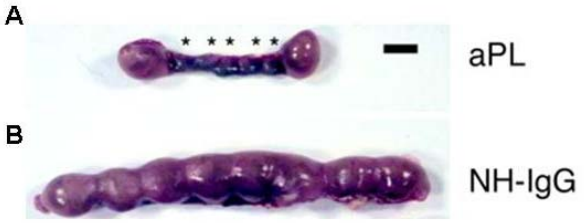

Figure 1-1: Murine model of APS.
Pregnant BALB/c mice were given aPL (10mg, intraperitoneally) or NHIgG (10mg, intraperitoneally) on days 8 and 12 of pregnancy. The uteri were harvested on day 15 of pregnancy. (A) There are two small amnionic sacs and 5 resorptions (*) in the uterus from an aPL treated mouse, (B) Mouse treated with NHIgG has normal size amnionic sacs and no resorption.

Role of Complement in aPL-mediated Fetal Loss

The complement system, that consists of over 30 proteins, is a highly sophisticated host defense system that acts to protect the host against infections activating the inflammatory response and causing tissue injury (Salmon et al., 2003). There are three complement pathways that make up the complement system:

1. Classical complement pathway- activated by antigen antibody complexes and unleashes effectors associated with humoral responses in immune-mediated tissue damage (Salmon et al., 2003).
2. Alternative pathway- initiated by the binding of activated complement components to the surface of pathogens and also on host tissues and
3. Lectin pathway- activated by the interaction of microbial carbohydrates with mannose binding proteins in the plasma and tissue fluids.

The 3 complement activation pathways converge on the C3 protein, which is cleaved to generate fragments C3a and C3b. C3a is an anaphylatoxin that binds to receptors on leukocytes and other cells leading to activation and release of soluble inflammatory mediators. C3b attaches to targets and following assembly of C5 convertase leads to cleavage of C5 to C5a and C5b. C5a is a potent inflammatory anaphylatoxic and chemotactic molecule that recruits and activates neutrophils and monocytes and mediates endothelial cell activation. Binding of C5b to target initiates assembly of C5b-9 membrane attack complex (MAC). The

MAC complex causes lysis of bacteria as well as human cells displaying foreign antigens and activates proinflammatory signaling pathways (Salmon et al., 2003).

Findings from animal models of aPL-mediated pregnancy loss have shown that complement factors C3 and C5 are essential proximal mediators of tissue injury (Girardi et al., 2003; Holers et al., 2002; Pierangeli et al., 2005). Intact complement regulation seems to be essential for normal pregnancies. Studies suggest that uncontrolled activation of the complement pathway leads to pregnancy failure even in the absence of aPL (Salmon et al., 2007). Blockade of the complement cascade *in vivo* with a C3 convertase inhibitor (Crry-Ig), a monoclonal antibody to C5 or a C5a receptor antagonist peptide reverses fetal loss and growth restriction in mice treated with aPL from human patients (Girardi et al., 2003; Holers et al., 2002; Salmon et al., 2007). Also, mice deficient in complement C3, C5 or C5a receptors are resistant to fetal loss by aPL (Salmon et al., 2007).

Figure 1-2 gives an overview of the pathway of aPL-mediated fetal damage caused by the activation of complement. (Girardi et al., 2006).

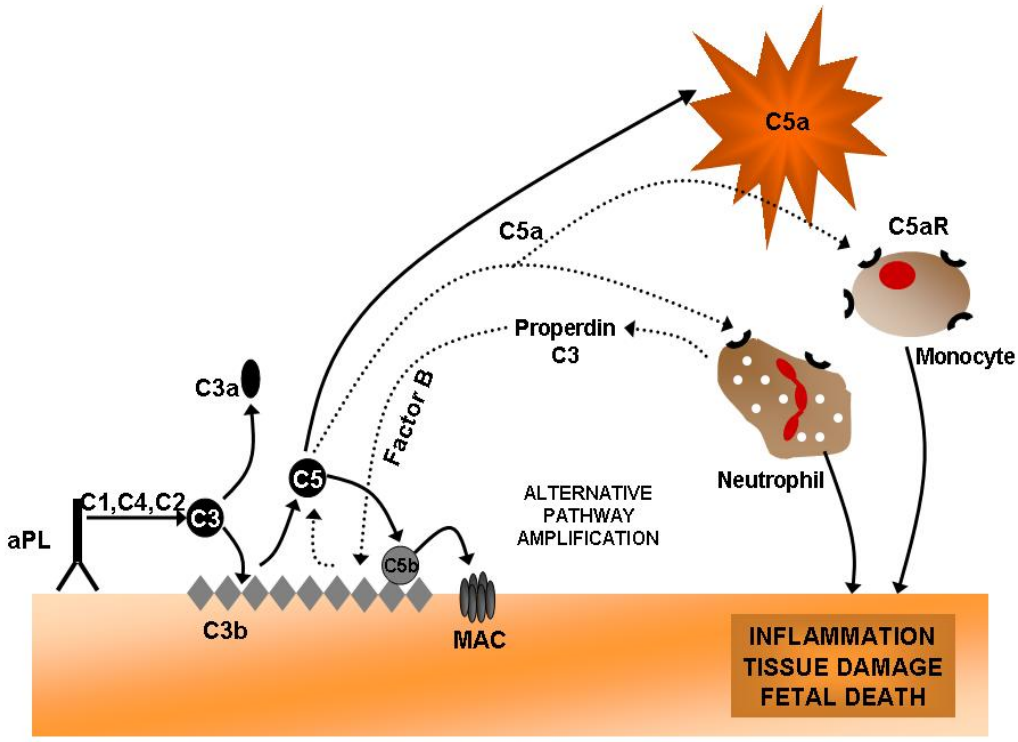

Figure 1-2: Mechanism of aPL-mediated fetal damage.
aPL are targeted to the placenta where they activate the classical complement pathway (C1 to C5 are components of the complement pathway) leading to C5a generation. C5a attracts and activates monocytes and neutrophils leading to release of inflammatory mediators. This may accelerate alternate pathway activation, creating a proinflammatory amplification loop leading to further activation via alternate pathway protein factor B and a serum protein properdin forming a complex that can cleave and activate C3 and generating additional C5a. This results in further influx of neutrophils, inflammation within the placenta and finally fetal injury. Binding of C5b to target membrane initiates the formation of the membrane attack complex (MAC) which also causes lysis of host cells displaying foreign antigen. Depending on the extent of damage either fetal death or fetal growth restriction occurs. (Modified from Girardi et al, 2006).

Heparin therapy might prevent obstetric complications in women with APS by blocking complement activation in placental tissues and also by preventing placental thrombosis (Salmon et al., 2007).

Cardiovascular Manifestations of APS

Valvular heart disease is the most common form of aPL-related cardiac involvement. Studies also support aPL as a risk factor for coronary artery disease and myocardial infarction (Sammaritano, 2005). Patients with aPL have higher risk of coronary artery bypass graft occlusion and an increased risk of atherosclerosis (Sammaritano, 2005). Atherosclerosis can be transferred between animals using autoantigen sensitized lymphocytes from immunized animals (Jara et al., 2003). aPL may stimulate thrombin formation on endothelium. Deposits of aCL (mainly IgG) and complement are found in the sub-endothelial connective tissue of deformed valves of APS patients (Tenedios et al., 2005). aPL correlate with atherosclerosis, and uptake of oxidized low density lipoprotein (oxLDL) by macrophages in the vessel wall that is decreased by β2GPI in normal subjects is blocked in the presence of anti –β2GPI antibodies. Thus, macrophage uptake of oxLDL is increased leading to accelerated atherosclerosis (Tenedios et al., 2005). There is an increase in carotid intima-medial thickness (IMT) and decrease in lumen diameter without atherosclerotic plaque in primary APS patients (Medina et al., 2003; Tenedios et al., 2005). Intracardiac thrombi have been identified in

the evaluation of emboli in primary APS patients (Tenedios et al., 2005). Increased occurrence of thrombosis present in APS patients may be associated with premature and accelerated atherosclerosis. Atherosclerosis in APS is mediated directly by proinflammatory and procoagulant activity of aPL on vascular endothelial cells or indirectly by immuno-inflammatory mechanisms underlying autoantibody-mediated thrombosis (Soltesz et al., 2007).

Mechanisms of aPL-mediated Platelet Dysfunction

Thrombocytopenia is a frequent feature in APS and studies have demonstrated binding of affinity-purified aPL to platelets (Harris et al., 1985; Hasselaar et al., 1990; Khamashta et al., 1988; Out et al., 1991; Shechter et al., 1998). aCL from APS patients enhances activation of platelets treated with suboptimal doses of adenosine diphosphate (ADP), thrombin, collagen or thrombin receptor agonist peptide (TRAP). aPL-β2GPI complexes increases production of thromboxin A_2 (TXA_2), a proaggregatory prostanoid in platelets (Vega-Ostertag et al., 2004). Phosphorylation of p38 Mitogen Activated Protein Kinase (p38MAPK) mediates aPL-induced activation of platelets *in vitro* (Vega-Ostertag et al., 2004). Dimeric F(ab')2 fragment of either aPL or β2GPI monoclonal antibody causes thrombus formation in an *in vivo* model (Jankowski et al., 2003; Vega-Ostertag et al., 2004). Dimeric β2GPI and whole blood spiked with patient-derived polyclonal anti-β2GPI antibodies increases platelet adhesion

to collagen (Lutters et al., 2003). A splice variant of apolipoprotein E receptor 2 (apoER2', a member of the low density lipoprotein receptor (LDLR) family of proteins) in platelets has been demonstrated to be involved in the binding of dimeric β2GPI to human platelets (Lutters et al., 2003).

Mechanisms of aPL-mediated Endothelial Dysfunction

Based on its critical role in thrombosis and inflammation, the endothelium has received considerable attention as a target for aPL action. Polyclonal aPL from patients or both polyclonal and monoclonal antibodies to β2GPI have been found to induce a pro-adhesive endothelial cell phenotype which is mediated by upregulation of adhesion molecules like E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1), and they also increase the secretion of proinflammatory cytokines such as interleukin-1β (IL-1β) and IL-6 and cause increased monocyte adhesion (Del Papa et al., 1997; Del Papa et al., 1995; Del Papa et al., 1998; George et al., 1998; Gharavi et al., 1999; Le Tonqueze et al., 1995; Pierangeli et al., 1999; Simantov et al., 1995). aPL also induce monocyte chemoattractant protein-1 (MCP-1) in endothelial cells, suggesting an initiating mechanism for monocyte-endothelial cell interaction (Cho et al., 2002). A pro-adhesive and a pro-inflammatory phenotype of endothelial cells is associated with the appearance of a pro-coagulant state (Nawroth and Stern, 1987). Endothelial derived microparticles, which are

membrane blebs from activated cells that contain cytoplasmic components and membrane components such as negatively charged phospholipids and cell surface receptors, are present in normal human blood and are increased in the plasma of patients with APS (Combes et al., 1999). Also, Pierangeli et al. have described an experimental model in which the passive infusion of aPL upregulates white blood cell (WBC) adhesion to endothelium and thrombus formation as observed by direct microscopic examination in mice (Pierangeli et al., 1999). aPL also cause upregulation of tissue factor expression in endothelial cells, which results in initiation of the extrinsic coagulation system (Amengual et al., 1998; Branch and Rodgers, 1993; Conti et al., 2003; Kornberg et al., 1994). aPL also upregulate endothelin-1 (ET-1) mRNA expression in cultured endothelial cells and increased levels of circulating ET-1 are found in APS patients, indicating that aPL may affect arterial tone and thrombotic arterial occlusion through upregulation of ET-1 (Amengual et al., 1998).

E-selectin upregulation by anti-β2GPI antibodies is dependent on NF-κB activation similar to that induced by proinflammatory cytokines or lipopolysaccharide (LPS) (Riboldi et al., 2003). Anti-β2GPI antibodies also cause endothelial cell dysfunction through a MyD88-dependent signaling pathway indicating a role for the Toll- like receptor family (TLR)-4 (Meroni et al., 2004; Raschi et al., 2003). Upregulation of pro-inflammatory, pro-coagulant and pro-

thrombotic factors contribute to endothelial cell dysfunction leading to the thrombotic manifestations of APS.

Molecules involved in aPL-mediated endothelial cell dysfunction

Although initially thought to recognize phospholipids directly, it is now apparent that aPL bind to phospholipid binding molecules like β2GPI and annexins (Galli et al., 1990; McNeil et al., 1990; Rand et al., 2004).

Beta 2 glycoprotein I (β2GPI)

β2GPI is a plasma protein present in the serum at a concentration of 200 μg/ml. It is a highly glycosylated single chain polypeptide of approximately 50kDa (Lozier et al., 1984) consisting of five highly conserved subunits (domain I-V) and binding of β2GPI to phospholipids membranes occurs through domain V (Hagihara et al., 1995; Hunt and Krilis, 1994; Hunt et al., 1993; Matsuura et al., 1995) (**Figure 1-3**).

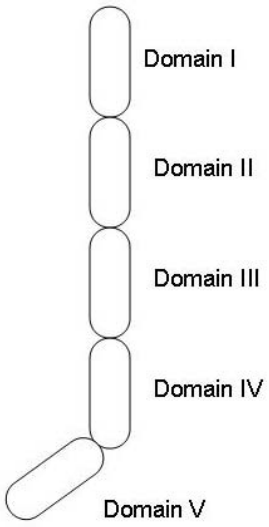

Figure 1-3: Structure of beta-2 glycoprotein I (β2GPI).
β2GPI consists of 5 domains (I –V). It binds to the endothelial cell surface through domain V.

β2GPI interacts with the surface membrane of platelets, endothelial cells and trophoblasts (La Rosa et al., 1994; McIntyre, 1992; Meroni et al., 1998; Schousboe, 1980). Antibodies directly reacting with β2GPI have been found in patients with APS (Del Papa et al., 1998; Matsuura et al., 1995; Roubey, 1998). Both polyclonal and monoclonal antibodies against β2GPI cause a proinflammatory and procoagulant phenotype in endothelial cells sustained by the upregulation of expression of adhesion molecules, and the synthesis and secretion of cytokines, chemokines, ET-1 and tissue factor (Meroni et al., 2001). aPL

mainly recognizes domain I of β2GPI, but some aPL also bind to other domains and to inter-domain regions (Gushiken et al., 2000).

In studies of β2GPI in mice, thrombin generation was impaired in knockout ($\beta2GPI^{-/-}$) mice compared to wild type ($\beta2GPI^{+/+}$) and heterozygous $\beta2GPI^{+/-}$ mice (Sheng et al., 2001). Fetal: placental weight ratio (measure of placental function) was decreased in $\beta2GPI^{-/-}$ mice but there was no effect on the length of gestation compared to wild type mice. Passive administration of either polyclonal aPL or monoclonal β2GPI antibodies had similar effect on pregnancy loss in $\beta2GPI^{+/+}$ vs. $\beta2GPI^{-/-}$ mice (Robertson et al., 2004). These studies suggest that mechanisms requiring molecules other than β2GPI are operative in aPL-mediated fetal loss.

Annexin A2

Annexin A2 belongs to a family of structurally related cell surface proteins known to bind PL in a Ca^{2+}-dependent manner (Rand, 1999; Raynal and Pollard, 1994). Annexins are ubiquitous proteins with structures characterized by a conserved C-terminal domain with Ca^{2+} binding sites and a variable N-terminal domain. Depending on Ca^{2+} concentration, they participate in a variety of membrane-related events such as exocytosis, endocytosis, apoptosis and the binding to cytoskeletal proteins (Camors et al., 2005). Annexin A2 greatly enhances the

catalytic efficiency of tissue plasminogen activator (t-PA)-mediated plasminogen activation on cell surfaces (Cesarman et al., 1994; Hajjar and Menell, 1997; Kassam et al., 1998a; Kassam et al., 1998b). Unstimulated endothelial cells bind β2GPI largely through a high-affinity interaction with annexin A2 expressed on the cell surface, and anti-annexin A2 antibodies directly cause endothelial cell activation of a similar magnitude as β2GPI antibodies. (Ma et al., 2000). Bivalent anti-annexin A2 F(ab')2 fragments also cause endothelial cell activation, whereas monomeric Fab fragments are not only ineffective in causing activation, but block activation induced by intact anti-annexin A2 antibodies and bivalent F(ab')2 fragments, as well as that caused by anti-β2GPI antibodies. Thus, a pathway has been proposed in which aPL /anti-β2 GPI antibody initiates endothelial activation by crosslinking or clustering of annexin A2 on the endothelial surface (Zhang and McCrae, 2005). The β2GPI- annexin A2 dependent pathway of endothelial cell activation is schematically represented in **Figure1-4**.

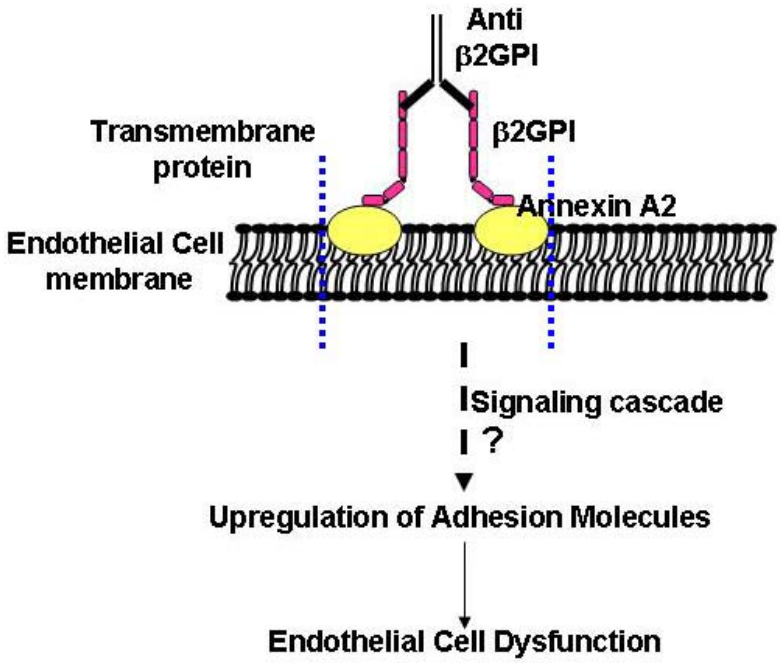

Figure 1-4: Endothelial cell dysfunction mediated by antibody/ β2GPI complexes and annexin A2.
Binding of antibody to β2GPI via the bivalent F(ab')2 region of the antibody, causes dimerization of β2GPI and binding to cell surface annexin A2. This complex causes upregulation of adhesion molecules via an unknown transmembrane protein. Upregulation of adhesion molecules leads to a dysfunctional endothelium.

Low density lipoprotein receptor (LDLR) family

The low density lipoprotein receptor (LDLR) family comprises a large number of genes, whose products contain a characteristic set of structural domains. Members of the LDLR family are involved in various cellular functions, including receptor-mediated endocytosis of a variety of ligands and

also as cell surface receptors in signal transduction pathways (Schneider and Nimpf, 2003).

The members of the LDL receptor family include LDLR, LDLR related protein-1 (LRP, LRP-1), megalin, very low density lipoprotein receptor (VLDLR), apolipoprotein E receptor type 2 (apoER2), LRP-3, LRP-4, LRP-5, LRP-6 and LR-32 (Schneider and Nimpf, 2003). These proteins have structurally and functionally defined modules including an O linked sugar domain, a transmembrane domain and a short cytoplasmic tail with one/ more short signals (NPxY) for receptor internalization via coated pits (Schneider and Nimpf, 2003). Some members of the LDLR family are schematically represented in **Figure1-5**.

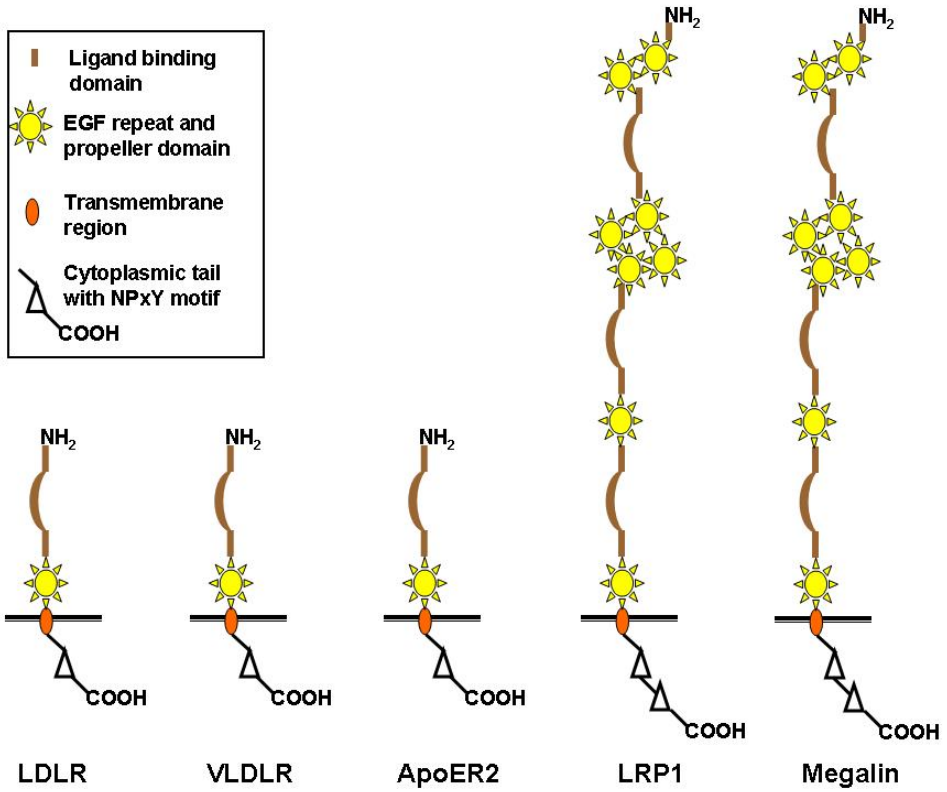

Figure 1-5: Schematic representation of the LDL receptor family.
The structural organization of some members of the LDL Receptor family including LDLR, VLDLR, apoER2, LRP1 and megalin are depicted. Modified from Beffert et al, 2004 (Beffert et al., 2004).

apoER2 and VLDLR have been recognized as signal transducers for the secreted glycoprotein reelin, which regulates neuronal positioning in laminated structures of the developing brain. These receptors are predominantly expressed in the central nervous system (May et al., 2005). However, different splice variants of apoER2 are also expressed in the endothelium (Korschineck et al.,

2001; Sacre et al., 2003). VLDLR is also expressed in the endothelium (Wyne et al., 1996). Apolipoprotein E and reelin are ligands for apoER2, and apolipoprotein E, reelin, lipoprotein lipase and tissue factor pathway inhibitor are ligands for VLDLR (May et al., 2005).

LRP/ LRP1 is a ubiquitously expressed protein that has a dual role in endocytosis and signal transduction (May et al., 2005). LRP1 is expressed in the renal endothelium and in mouse brain microvessel endothelial cells (MvEC) (Caron et al., 2005; Fears et al., 2005). Ligands for LRP1 include apolipoprotein E, chylomicron remnants, α2-macroglobulin, amyloid precursor protein (APP), tissue plasminogen activator (tPA), protease/ protease inhibitor complexes, lipoprotein lipase, platelet derived growth factor (PDGF) and TGF-β (May et al., 2005). LRP1 knockout mice are embryonic lethal, but studies in tissue-specific knockout animals and using neutralizing antibodies have helped elucidate its functions. In the blood vessel, LRP1 is required for tissue plasminogen (tPA) activity and influences vessel development and integrity (May et al., 2005). Megalin is another multifunctional member of the LDLR family and has a role in endocytosis and transport (May et al., 2005). Ligands for megalin include apolipoprotein B, apolipoprotein E, apolipoprotein J, apolipoprotein H, albumin, cubulin, retinol binding protein, vitamin D binding protein, sonic hedgehog and bone morphogenic protein-4 (BMP-4) (May et al., 2005). Megalin is expressed in renal endothelium (Caron et al., 2005).

Dimeric β2GPI can bind to members of the LDLR family including apolipoprotein E receptor 2' (apoER2', splice variant of apoER2 in platelets), very low density lipoprotein receptor (VLDLR), LDLR related protein (LRP), and megalin (Pennings et al., 2006). Using surface plasma resonance (SPR) analysis and immunosorbent assays, Pennings et al. demonstrated that a complex of monoclonal β2GPI antibody and plasma β2GPI interacts with LRP, megalin, apoER2' and VLDLR but does not interact with low density lipoprotein receptor (LDLR). The interaction of β2GPI with LDLR family members was inhibited by addition of an inhibitor of the LDL receptor family, receptor associated protein (RAP). Domain V of β2GPI is required for the interaction of β2GPI with LRP, megalin, apoER2' and VLDLR whereas domain I and domain II of β2GPI are required for binding to apoER2' and VLDLR to a lesser extent (Pennings et al., 2006).

Figure 1-6 depicts a summary of the pathway leading to aPL-mediated endothelial cell dysfunction and increased thrombosis, inflammation and tissue damage. Binding of aPL to target proteins like β2GPI and via unknown receptor/ adaptor proteins leads to increased expression of adhesion molecules on endothelial cells. The signaling pathway from aPL binding to the endothelium to increased adhesion molecule expression is unknown.

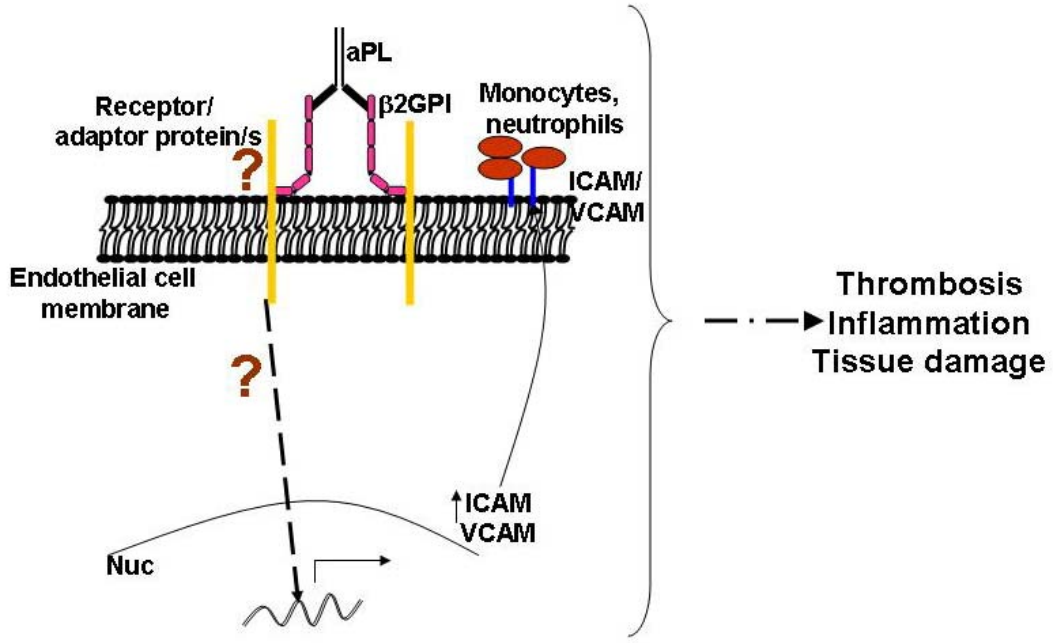

Figure 1-6: Schematic representation of the effect of aPL on the endothelium. Binding of aPL to target proteins (like β2GPI) on endothelial cells causes increase in the expression of adhesion molecules like intracellular adhesion molecule (ICAM) and vascular cellular adhesion molecule (VCAM) (shown in blue) and also other proinflammatory cytokines, tissue factor and endothelin-1 (not depicted). This effect of aPL yields a dysfunctional endothelium, leading to increased thrombosis, inflammation and tissue damage.

Endothelial Nitric Oxide Synthase (eNOS)

Nitric oxide (NO) is a gaseous free radical that is recognized as a key determinant of vascular health. NO regulates several physiologic processes including vascular tone, vascular permeability, endothelial-monocyte interaction, vascular cell proliferation, and the anti-thrombotic properties of the endothelium

(Voetsch et al., 2004). These functions converge to maintain normal endothelial phenotype and an anti-thrombotic intravascular environment. As a consequence, insufficient bioavailable NO has widespread pathophysiological implications. Impaired NO bioavailability represents a central feature of endothelial dysfunction in the earliest stage of atherosclerotic process, and also contributes to the pathogenesis of acute vascular syndromes by predisposing to plaque rupture and intravascular thrombosis. The intact endothelium normally prevents platelet adhesion to the extracellular matrix, whereas dysfunctional endothelial cells develop properties that render them adhesive for platelets. NO prevents platelet activation by activating guanylyl cyclase and preventing platelet aggregation (Loscalzo, 2001). NO also inhibits platelet recruitment to the thrombus and attenuates platelet thromboxane synthesis (Freedman et al., 1999).

Because of its potent bioreactivity and high diffusability, NO production by the enzyme nitric oxide synthase (NOS) is under tight control to dictate specificity of its signaling and to limit toxicity to other cellular compartments. NO production from all of the three NOS isoforms, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) is controlled by a variety of transcriptional, translational and posttranslational mechanisms.

The endothelial NOS isoform, eNOS is the enzyme responsible for NO generation in the vascular wall. eNOS is acutely activated by agonists of diverse receptors and by physical stimuli including shear stress and varying oxygen

availability. NO generated by eNOS regulates blood pressure, platelet aggregation, leukocyte adherence and vascular smooth muscle cell mitogenensis. Decreased NO production by the endothelium has been implicated in systemic and pulmonary hypertension and also in other vascular disorders including atherosclerosis (Shaul, 2002). The role of eNOS in normal endothelial function is depicted in **Figure 1-7**.

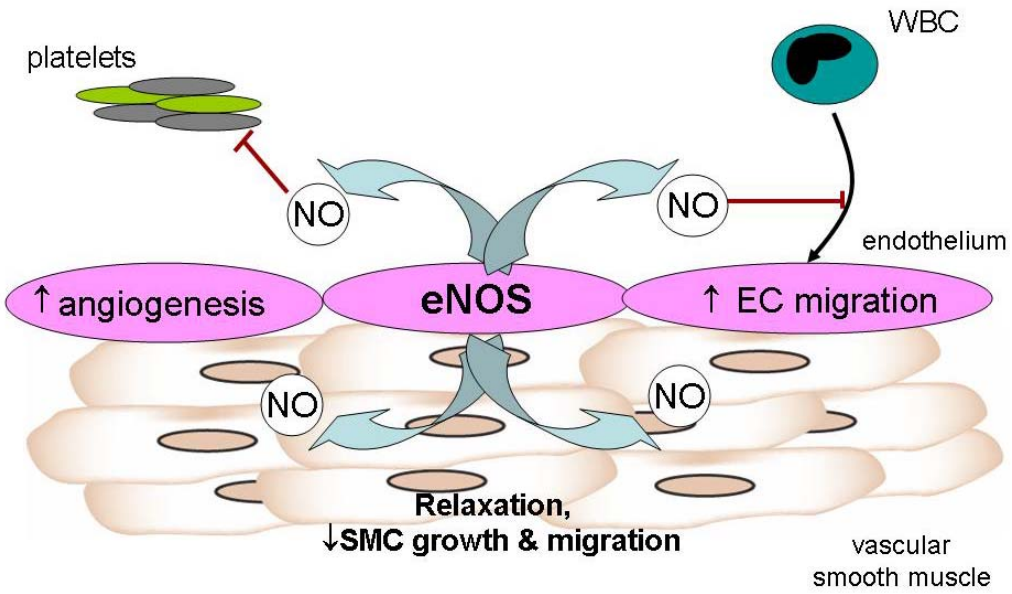

Figure 1-7: eNOS and normal endothelial function.
Generation of nitric oxide (NO) by eNOS in the endothelium causes increased endothelial cell migration and increase in angiogenesis. NO also causes the relaxation of the underlying vascular smooth muscle cells and causes decreased smooth muscle cell growth and migration. NO also blocks adhesion of leucocytes to the endothelium and also blocks platelet aggregation.

eNOS is also the predominant NOS isoform expressed in the platelets. Platelet-derived NO may act in an autocrine manner exerting its effects within the same cell in which it is produced as opposed to endothelium-derived NO which also diffuses to act on adjacent cells to exert its effects in a paracrine manner (Gkaliagkousi et al., 2007). Primary platelet aggragation is mainly due to lack of endothelial NO whereas platelet-derived NO inhibits recruitment of activated platelets to the growing thrombus (Gkaliagkousi et al., 2007). Heterotypic aggregation between leukocytes and platelets, particularly monocytes and platelets, is an early marker of platelet activation implicated in the progression of atherosclerosis and thrombosis. Platelet-derived NO inhibits heterotypic aggregation between leukocytes and platelets *in vitro* (Gkaliagkousi et al., 2007). Platelet-derived NO and endothelium-derived NO both block platelet adhesion to endothelial cells.

Structure of eNOS Protein

eNOS is a bidomain protein, in which a central calmodulin-binding motif separates an oxygenase (COOH-terminal) from a reductase (NH_2-terminal) domain (Kone, 2000). The oxygenase domain contains a iron protoporphyrin IX (haem) active site and a binding site for (6R)-5,6,7,8-tetrahydrobiopterin (BH_4). The reductase domain contains an electron transfer domain that binds to flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). eNOS is dimeric

in its active form and requires binding of calmodulin and BH4 for activity. Transient elevations in intracellular free Ca^{+2} levels trigger calmodulin binding which serves as an allosteric modulator of eNOS. eNOS contains an autoinhibitory loop in the middle of the FMN binding sub-domain that destabilizes calmodulin binding at low calcium concentrations and inhibits electron transfer from FMN to haem in the presence of Ca^{+2}/ calmodulin. The domain structure of eNOS is depicted in **Figure 1-8**.

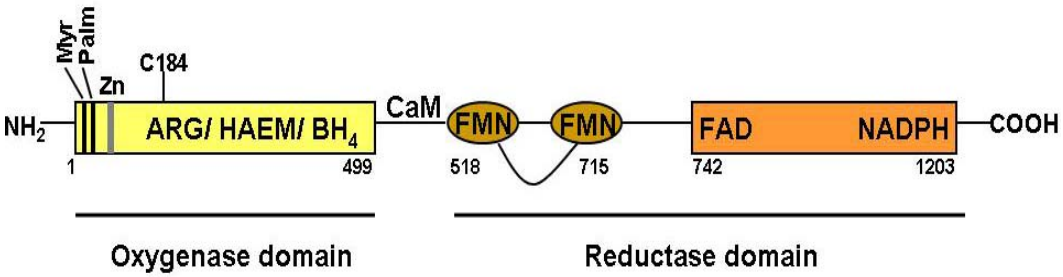

Figure 1-8: Domain structure of eNOS.
Oxygenase and reductase domains are depicted by solid boxes and the amino acid residue at the start and end of each domain is numbered. Myristoylation (**Myr**), palmitoylation (**Palm**) and the location of the Zn ligating cysteines (**Zn**, grey) is shown. **C184** is the cysteine residue that ligates the haem and the CaM binding site. The autoinhibitory loop between the FMN regions is indicated.
Modified from Alderton et al, 2001 (Alderton et al., 2001).

NO is synthesized from L-arginine, molecular oxygen and NADPH through a five-electron oxidation step. After binding of calcium loaded calmodulin to eNOS, electrons are donated by NADPH at the reductase domain which are shuttled through the calmodulin-binding domain toward the haem-containing oxygenase domain which results in the formation of free radical NO, L-citrulline and NADP. (Alderton et al., 2001; Govers and Rabelink, 2001; Kone, 2000).

Modulation of eNOS Activity

eNOS activity is modulated by its expression and also by a complex combination of enzyme localization, protein-protein interactions and signal transduction events involving calcium mobilization and a variety of phosphorylation events.

Modulation of eNOS activity by expression

eNOS is expressed in the endothelium and is also found in the bronchial and proximal bronchiolar epithelium (Shaul, 2002). The eNOS promoter contains proximal elements like the Sp1 and GATA motifs but does not contain a TATA box typical of a constitutively expressed protein. The human eNOS promoter also contains binding sites for transcription factors like activator protein-1 (AP-1), activator protein-2 (AP-2), Ets family members, myc-associated Zn finger protein

(MAZ), nuclear factor-1 (NF-1), NF-IL6, NF-κB, p53, PEA3 and YY1 as well as CACCC, CCAAT, heavy metal, acute phase response, shear stress, cAMP response, retinoblastoma control, interferon-γ response and sterol regulatory *cis* elements (Fleming and Busse, 2003).

eNOS expression is upregulated by shear stress by increased eNOS transcription and also by increasing eNOS mRNA stability (Govers and Rabelink, 2001; McAllister and Laughlin, 2006). Sp1 has been shown to be involved in lysophosphatidylcholine (lysoPC)-induced upregulation of eNOS transcription (Cieslik et al., 1999; Govers and Rabelink, 2001). AP-1 activation increases eNOS expression in the presence of immunosuppressive drugs like cyclosporine A (Govers and Rabelink, 2001). eNOS transcription is also upregulated by cyclic strain, hydrogen peroxide (H_2O_2), estrogen, vascular endothelial growth factor (VEGF), insulin, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and low concentrations of oxidized LDL (ox-LDL) (Govers and Rabelink, 2001; Searles, 2006). VEGF, H_2O_2, increased cell growth and shear stress induce eNOS upregulation by increasing eNOS mRNA stability (Searles, 2006). eNOS expression is lowered by tumor necrosis factor-α (TNF-α), erythropoietin, hypoxia, and high concentrations of ox-LDL (Govers and Rabelink, 2001). C-reactive protein (CRP) also causes a decrease in eNOS expression (Venugopal et al., 2002). eNOS expression is also regulated by NO. NO decreases eNOS expression by a negative feedback mechanism via a cGMP-mediated process

(Govers and Rabelink, 2001). Some stimuli that decrease eNOS mRNA stability include lipopolysaccharides (LPS, endotoxins), hypoxia, ox-LDL and TNF-α (Govers and Rabelink, 2001).

Modulation of eNOS activity by localization

Functional eNOS is primarily associated with the plasma membrane and is acutely regulated by extracellular factors (Hecker et al., 1994). eNOS has been shown to be localized in caveolae, which are specialized plasma membrane microdomains enriched in glycosphingolipids, sphingomyelin, cholesterol and lipid-anchored membrane proteins (Shaul, 2002; Shaul et al., 1996). Caveolae also contain a variety of signal transduction molecules, including G protein coupled receptors, muscarinic acetylcholine receptor, G-proteins and molecules involved in the regulation of intracellular calcium homeostasis such as the plasma membrane calcium pump and protein kinase C (Chang et al., 1992; Chang et al., 1994; Conrad et al., 1995). Localization of eNOS to the caveolae correlates with enzyme activity, as NOS activity is 7-fold greater in the plasma membrane fraction than in the cytosol and within the plasma membrane fraction, NOS activity is non-existent in the non-caveolae fraction whereas it is 9-10 fold greater in the caveolae membranes compared to whole plasma membrane (Shaul, 2002; Shaul et al., 1996). eNOS has also been localized using immunogold microscopy to the caveolae microdomain (Shaul, 2002; Shaul et al., 1996). Myristoylation of

eNOS at the glycine residue in position 2 and palmitoylation of cysteines at positions 15 and 26 targets the enzyme to the caveolae (Lamas et al., 1992; Robinson and Michel, 1995; Shaul et al., 1996). A mutant of eNOS that cannot be palmitoylated is targeted to the Golgi apparatus whereas a mutant that cannot be myristoylated is distributed throughout the cell (Liu et al., 1996; Liu et al., 1997; Shaul, 2002; Sowa et al., 1999).

The specialized lipid environment in the caveolae is critical for eNOS targeting to this microdomain and its function (Shaul, 2002). Oxidized LDL (ox-LDL) inhibits vasodilation by NO and treatment of endothelial cells with ox-LDL causes eNOS and caveolin to translocate to an internal membrane fraction (containing endoplasmic reticulum, Golgi apparatus, mitochondria, and other intracellular organelles) from the caveolae and plasma membrane fraction. Ox-LDL also attenuates enzyme activation by acetylcholine (Blair et al., 1999; Shaul, 2002). Ox-LDL causes a depletion of caveolae cholesterol and this change in the lipid environment leads to the redistribution and attenuation of eNOS (Blair et al., 1999; Shaul, 2002).

Modulation of eNOS Activity by Ca^{+2} and Protein-Protein Interactions

eNOS activity under certain conditions is regulated by changes in the cytosolic calcium concentration and can be activated by hormones that can cause an increase in intracellular calcium levels including bradykinin, estradiol,

histamine, serotonin and VEGF (Govers and Rabelink, 2001). Calmodulin (CaM) binds calcium, and the calcium-calmodulin complex binds to eNOS leading to increased eNOS enzymatic activity. Mechanistically, CaM binding to the CaM-binding motif is thought to displace an autoinhibitory loop on eNOS, facilitating NADPH-dependent electron flux from the reductase to the oxygenase domain of the protein (Fleming and Busse, 2003).

Interaction of eNOS with different proteins plays an important role in the activity of the enzyme. eNOS is co-immunoprecipitated with caveolin-1 in endothelial cells (Feron et al., 1996; Garcia-Cardena et al., 1996). Overexpression and *in vitro* studies have further shown that caveolin-1 binds to the eNOS oxygenase domain and inhibits eNOS activity (Garcia-Cardena et al., 1997; Ju et al., 1997; Michel et al., 1997a; Michel et al., 1997b). Calcium-calmodulin binding may disrupt the interaction between eNOS and caveolin and thereby cause activation of the enzyme (Michel et al., 1997a).

Stimulation of endothelial cells by histamine, vascular endothelial growth factor (VEGF) or shear stress causes binding of heat shock protein 90 (Hsp90) to eNOS leading to allosteric activation of the enzyme (Garcia-Cardena et al., 1998). A 34-kDa protein, NOSIP (eNOS interacting protein), has also been identified that can bind to the C-terminus of the eNOS oxygenase domain and results in attenuation of enzyme activity by translocating it from the caveolae to intracellular sites (Dedio et al., 2001; Shaul, 2002). Activity of eNOS is also

regulated by its association with the C-terminal domain of G protein-coupled receptors like the bradykinin B2 receptor, which is mobilized to caveolae upon agonist stimulation (Ju et al., 1998; Shaul, 2002).

Modulation of eNOS activity by kinases and phosphatases

eNOS is phosphorylated on serine, threonine and tyrosine residues (Fleming and Busse, 2003). Most studies have been carried out on the functional consequences of phosphorylation of a serine residue (S1177 in human eNOS and S1179 in bovine eNOS) in the reductase domain and a threonine residue (T495 in human eNOS and T497 in bovine eNOS) present within the CaM-binding domain (Fleming and Busse, 2003).

S1177 is not phosphorylated in unstimulated endothelial cells but is rapidly phosphorylated by different agonists like VEGF, estrogen, insulin and HDL (Fleming and Busse, 2003; Mineo et al., 2003). The serine/ threonine kinase Akt (Protein kinase B, PKB) phosphorylates eNOS S1177 *in vitro* and *in vivo* leading to enzyme activation (Fulton et al., 1999; Gallis et al., 1999). When S1177 is phosphorylated, the dissociation of calmodulin from eNOS is reduced and this leads to an increase in NO production (McCabe et al., 2000). T495 is constitutively phosphorylated in endothelial cells and is a negative regulatory site (Fleming and Busse, 2003). More calmodulin binds to eNOS when T495 is dephosphorylated (Fleming et al., 2001).

Other than Akt, ERK1 and ERK2 as well as cyclic nucleotide dependent kinases PKA and PKG have also been reported to affect eNOS activity (Fleming and Busse, 2003). The protein phosphatases (PP) PP1 and PP2A play distinct roles in the regulation of eNOS phosphorylation and activation (Fleming and Busse, 2003). PP1 dephosphorylates T495 of eNOS and inhibition of PP1 results in hyperphosphorylation of T495, which inhibits eNOS activity (Fleming et al., 2001). PP2A is the phosphatase that dephosphorylates S1177 of eNOS (Fisslthaler et al., 2000; Michell et al., 2001). PP2A also dephosphorylates Akt and inactivates it (Andjelkovic et al., 1996).

Figure 1-9 is a schematic depicting the different mechanisms involved in the regulation of eNOS enzymatic activity.

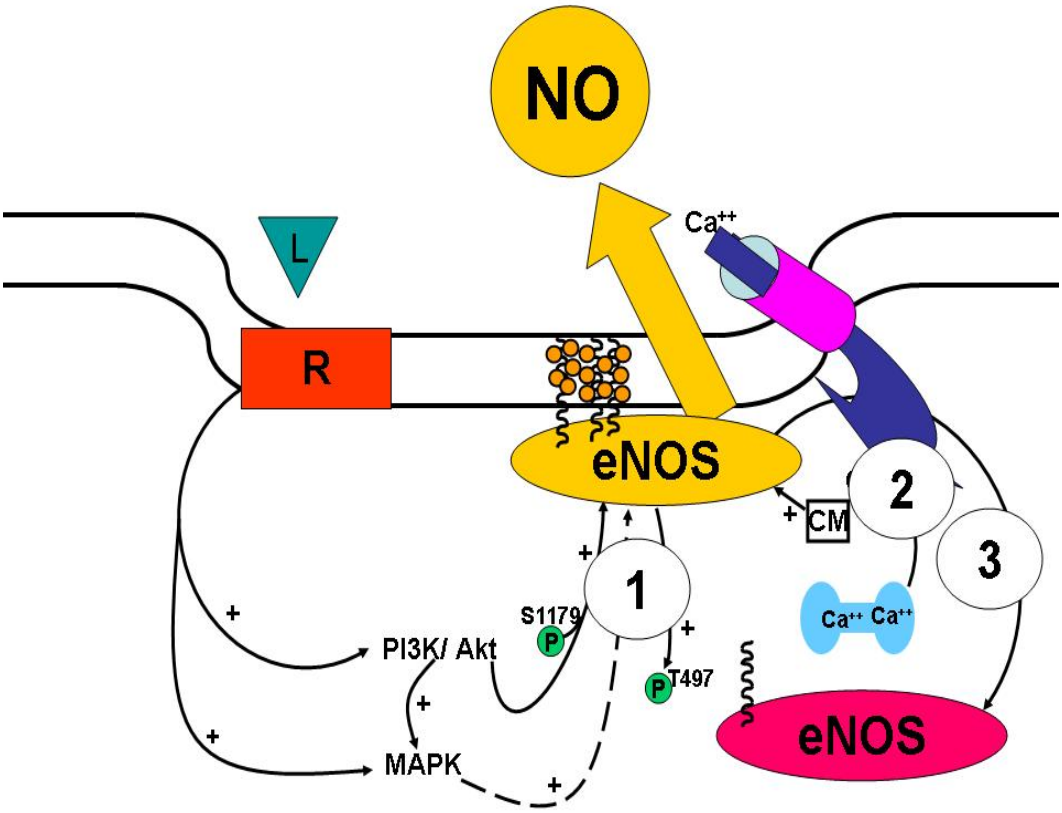

Figure 1-9: Mechanisms of eNOS modulation.
Activation of eNOS by different ligand (L) and receptor (R) pairs in endothelial cell caveolae is influenced by a combination of 3 primary processes. These are (1) phosphorylation events (S1179 phosphorylation, T497 dephosphorylation) and MAPK activation, (2) Calcium influx and/or calcium release from internal stores, which increases the association between calmodulin (CM) and eNOS, and (3) sub-cellular localization of eNOS (eNOS is active at the plasma membrane).

Overall Hypothesis

aPL causes endothelial cell dysfunction by recognizing molecules on the surface of the endothelial cells, predominantly the plasma glycoprotein β2GPI. β2GPI through unknown receptor/ adaptor proteins and unknown signaling pathways leads to increased adhesion molecule expression on the surface of the endothelial cells. This causes adhesion of monocytes and platelets to the endothelium and finally leads to increased thrombosis, inflammation and tissue damage. Endothelium-derived NO helps maintain the endothelium in an antiatherogenic state and inhibits monocyte adhesion, platelet aggregation and thrombus formation. We hypothesized that aPL causes endothelial cell dysfunction via blunting NO production by eNOS or NO bioavailability. In the following set of studies we determined the role of eNOS/ NO is aPL-mediated endothelial dysfunction both *in vitro* and *in vivo*. We also determined the effect of aPL on the activity of eNOS and the mechanisms involved. In addition, we investigated the involvement of β2GPI and LDL receptor family members in aPL-mediated endothelial dysfunction.

Chapter 2

Effect of aPL on eNOS

Introduction

Patients with antiphospholipid syndrome (APS) have an increased risk of coronary artery disease and myocardial infarction. The endothelium is a critical direct target of aPL, which causes increase in endothelial adhesion molecule expression leading to enhanced monocyte adhesion (Del Papa et al., 1995; Pierangeli et al., 1999; Riboldi et al., 2003; Simantov et al., 1995). The mechanisms by which aPL alters endothelial cell function and monocyte adhesion are unknown.

NO is a key determinant of vascular health regulating several physiological processes including thrombosis, interaction of endothelium and monocytes, endothelial cell migration and proliferation, vascular permeability and smooth muscle cell growth and migration. Impaired NO bioavailability represents a central feature of endothelial dysfunction in vascular thrombosis and is important in atherogenesis. eNOS is the endothelial isoform of nitric oxide synthase and is the primary source of bioavailable NO in the vasculature. eNOS generates NO by the conversion of L-arginine to L-citrulline. In this chapter, we have investigated the mechanisms underlying aPL-mediated endothelial dysfunction. In cell culture, we have tested the hypothesis that aPL-mediated

increases in monocyte adhesion to the endothelium are related to changes in NO bioavailability. We have also tested the hypothesis that aPL attenuates the activity of eNOS. Studies were also performed in mice to determine if the aPL-mediated changes in eNOS activity are also operative *in vivo*.

Methods

Cell Culture: Bovine aortic endothelial cells (BAEC) were harvested from fresh aortas from cattle less than 30 months of age. The aortas were transported from a local slaughterhouse in cold RPMI 1640 with antibiotics and antimycotics. After extensive washing of the outside and inside of the aortas with cold phosphate-buffered saline, the lumens were incubated in 2.5 mg/ml collagenase D in RPMI 1640 at room temperature for 8 min. Cells were collected and plated in gelatin-coated flasks in EGM-2 medium (Cambrex Bioscience) with 5% fetal bovine serum (FBS) (Sigma). The cells maintained a cobble-stone-like morphology and demonstrated uptake of DiL-labeled acetylated low density lipoprotein (DiL-ac-LDL) through several passages. BAEC were cultured in EGM-2 medium with 5% FBS, and used between passages 5 and 9. Human Aortic Endothelial Cells (HAEC) were purchased from Cambrex Bioscience and cultured in EGM-2 medium (Cambrex Bioscience) with 2% fetal bovine serum (Sigma). MFLM-91U mouse endothelial cell line was provided by Dr. Ann Akeson (Childrens Medical Center, Cincinnati, Ohio) and cultured in Ultraculture media (Cambrex). U937 monocytes (human histiocytic lymphoma, American Type Culture Collection) were grown in RPMI 1640 Medium (Sigma) containing 10% FBS.

Antibody Preparation: Human polyclonal antiphospholipid antibodies (aPL) were isolated from patients with APS, which were characterized as having high-titer aPL Ab (>140 GPL U), thromboses, and/or pregnancy losses (Girardi et al., 2003; Holers et al., 2002; Wilson et al., 1999). Normal human IgG (NHIgG) was obtained from healthy non-autoimmune individuals. The IgGs were purified by affinity chromatography using protein G–Sepharose chromatography columns (Amersham Biosciences) (Holers et al., 2002). Endotoxin was removed using Centriprep ultracentrifugation devices (Millipore), and lack of endotoxin was confirmed using the *Limulus* amebocyte lysate assay (Girardi et al., 2003). aPL and NHIgG were provided by Dr. J.E. Salmon of Cornell University.

Monocyte Adhesion Assay: The adhesion of U937 monocytes to monolayers of BAEC was evaluated as described previously (Kumar et al., 2007; Mineo et al., 2005). Confluent BAEC were exposed to LPS (100 ng/ml) for 18h in the absence or presence of other treatments (Ach, 10 μmol/L; nitro-L-arginine methyl ester, L-NAME, 2 mmol/L; aPL, 100 μg/ml; NHIgG 100 μg/ml; S-nitroso-N-acetyl-D,L-penicillamine, SNAP, 20 μmol/L). U937 monocytes ($1x10^6$ cells/ dish) were added and incubated with BAEC under rotating conditions (63 rpm) at 21°C for 15 min, nonadhering cells were removed by gentle washing with PBS, cells were fixed with 1% paraformaldehyde and the number of adherent cells were counted

in triplicate per 20X magnification field using an inverted microscope (Zeiss Axiovert 100M).

eNOS Activation Assay: eNOS activation was assessed in intact cells by measuring the conversion of [^{14}C]-L-arginine to [^{14}C]-L- citrulline, described in detail in reference (Pace et al., 1999). Endothelial cells were cultured in 6-well plates to 70-80% confluency. Briefly, cells were washed with 600 μl of PBS twice. Cells were preincubated with human polyclonal antiphospholipid antibodies (aPL, 100μg/ml) or human polyclonal normal IgG (NHIgG, 100 μg/ml) for 15 min in 600 μl PBS. Incubation with agonist was carried out in the presence of the antibody in 600 μl PBS containing [^{14}C] L-arginine for 15 minutes. The reaction was terminated by adding 750 μl of 15mM EGTA/ 200mM Hepes at pH 5.5 to all wells. The cells were frozen in liquid nitrogen and thawed at 37°C, repeating the freeze-thaw cycle twice. Cells were scraped off the bottom of the plate and 600 μl from each well was eluted over washed Sulpeco Dowex 50WX8 (SIGMA) columns and collected in 20 ml scintillation vials (Research Products International). 1 ml of Stop Solution I was eluted over the column after the sample fully entered the column. 20 ml of Universol^{TM} scintillation cocktail (MP Biomedical) was added to each vial and shaken till the solution was clear.

The vials were then counted in a LS6500 multipurpose scintillation counter (Beckman Coulter).

eNOS enzyme stimulation was assessed in PBS containing 120 mmole/L NaCl, 4.2 mmole/L KCl, 2.5 mmole/L $CaCl_2$, 1.3 mmole/L $MgSO_4$, 7.5 mmole/L glucose, 10 mmole/L Hepes, 1.2 mmole/L Na_2HPO_4, and 0.37 mmole/L KH_2PO_4 at pH 7.4. eNOS agonists tested were VEGF (100 ng/ml) and acetylcholine (Ach, 10 μmol/L). Dowex resin (Tris form) columns were prepared as follows: 2 ml Dowex resin slurry (pH 7.5 in the resin bed at room temperature) was added to polyprep prefilled chromatography columns (BioRad) and the effluent drained out. The columns were then washed with 2ml of 2mM EDTA/ 2mM EGTA/ 40mM Hepes, pH 5.5 (Stop Solution I).

Carotid Artery Vascular Conductance: To determine if aPL alters eNOS activity in vivo, Ach-mediated increases in carotid vascular conductance were measured before and after intravenous (I.V.) administration of aPL (2mg) or NH-IgG (2mg). 14 to 16 week old male C57BL/6 mice were used. Mice were anesthetized with tiletamine (4mg/kg), zolazepam (4 mg/kg), and zylazine (20mg/kg) given intraperitoneally (IP). Once catheters were placed in the right jugular vein and common carotid artery, the mice were switched to inhalation anesthesia (1% isoflurane), which was used continuously for the remainder of the experiment. 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 intravenous injections of acetylcholine (4-64 ng in a volume of 1-16 μl) were evaluated sequentially at baseline, 60 min after injection with either aPL (2mg IV) or NH-IgG (2mg IV) and 10 min after treatment with L-NAME (10 mg/kg IV). Six mice were studied per group.

Statistical Analysis: Data are expressed as mean +/- SEM. Student t-tests or ANOVA was used to assess differences between 2 groups or among more than 2 groups respectively. Significance was set at $P< 0.05$.

Results

aPL and monocyte adhesion to endothelial cells

aPL alters endothelial cell function (Amengual et al., 1998; Branch and Rodgers, 1993; Conti et al., 2003; Del Papa et al., 1997; Del Papa et al., 1995; Pierangeli et al., 1999; Riboldi et al., 2003; Simantov et al., 1995). We first determined the role of eNOS in the modulation of monocyte adhesion to the endothelium. Compared to control conditions (**Figure 2-1A**), treatment of BAEC with lipopolysaccharide (LPS) for 18 hours caused a marked increase in monocyte adhesion to the monolayer (**Figure 2-1B**). To determine if eNOS activation can reverse this adhesion of monocytes to the endothelium, BAEC were treated with LPS plus the eNOS agonist, Ach for 18 hours. Treatment with Ach reversed the effect of LPS on monocyte adhesion to BAEC (**Figure 2-1C**). This effect is specific to eNOS activation as the eNOS antagonist L-NAME reversed the lessening of monocyte adhesion by Ach (**Figure 2-1D**).

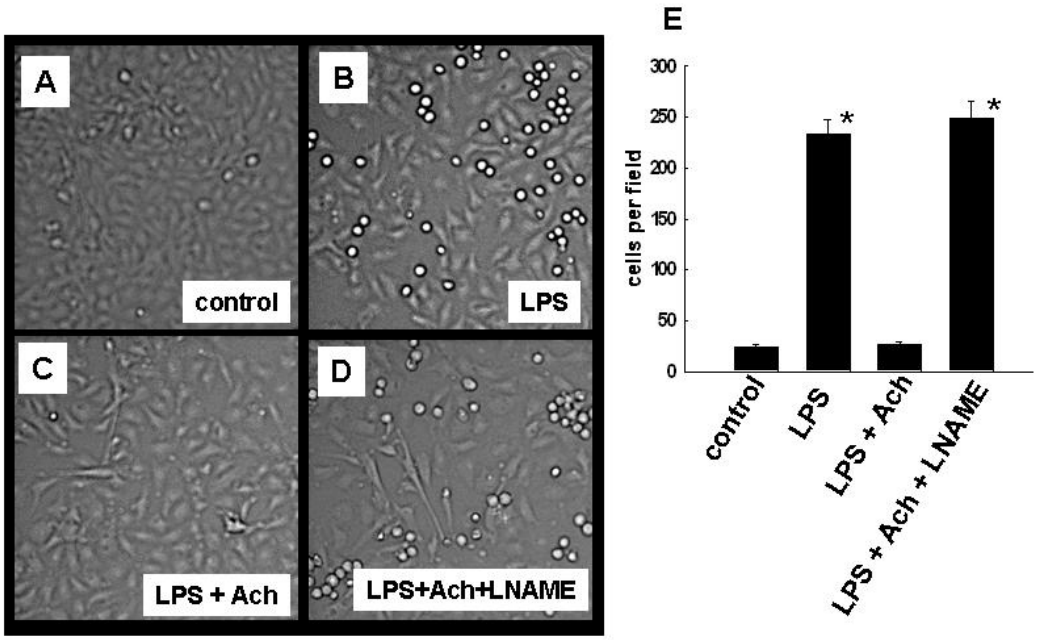

Figure 2-1: eNOS activation reverses LPS-mediated increases in monocyte adhesion to endothelial cells.
Monocyte adhesion to bovine aortic endothelial cells (BAEC) was evaluated under the following conditions: (**A**) control, (**B**) LPS treatment (100ng/ml), (**C**) LPS + Acetylcholine (Ach, 10μmol/L), and (**D**) LPS +Ach + nitro-L-arginine methyl ester (LNAME, 2mmol/L). Endothelium display cobblestone morphology and monocytes are circular and luminescent in appearance. (**E**) Graph represents summary data. In all graphs, values are mean $\pm$ SEM and n=3 unless otherwise indicated, and findings were replicated in 3 independent cell culture experiments. * P <0.001 vs. control.

To determine if aPL affects monocyte adhesion to endothelium, BAEC were treated with LPS or NHIgG (100 μg/ml) or aPL (100 μg/ml) for 18 hours and monocyte adhesion to the BAEC monolayer were evaluated. Compared to control conditions (**Figure 2-2A**), treatment with LPS caused a marked increase

in monocyte adhesion to BAEC (**Figure 2-2B**). Treatment of BAEC with NHIgG had no effect on monocyte adhesion (**Figure 2-2C**). In contrast, treatment with aPL increased monocyte adhesion to BAEC (**Figure 2-2D**).

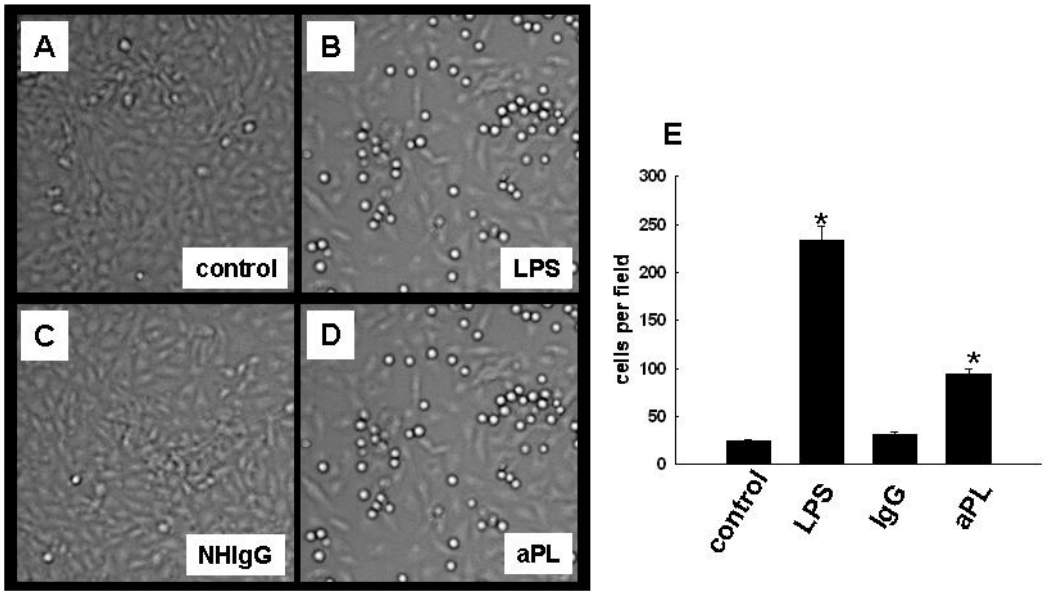

Figure 2-2: aPL causes increased monocyte adhesion to endothelial cells. Monocyte adhesion to BAEC was evaluated under the following conditions: (**A**) control, (**B**) LPS (100ng/ml), (**C**) NHIgG (100μg/ml), and (**D**) aPL (100μg/ml). (**E**) Graph represents summary data. ***** P <0.001 vs. control.

To determine if aPL actions on the endothelium are related to changes in bioavailable NO, BAEC were treated for 18 hours with different reagents and the impact on monocyte adhesion was evaluated. Compared to control conditions (**Figure 2-3A**), LPS caused a marked increase in monocyte adhesion (**Figure 2-**

3B) to BAEC. This was reversed by the eNOS agonist acetylcholine (**Figure 2-3C**). Whereas NHIgG had no effect on acetylcholine-mediated decreases in monocyte adhesion (**Figure 2-3D**), aPL reversed the effect of acetylcholine (**Figure 2-3E**) on adhesion. The impact of aPL on monocyte adhesion was reversed by the NO donor, SNAP (**Figure 2-3F**). These findings indicate that aPL-induced increases in monocyte adhesion to endothelium are due to decreased bioavailable NO.

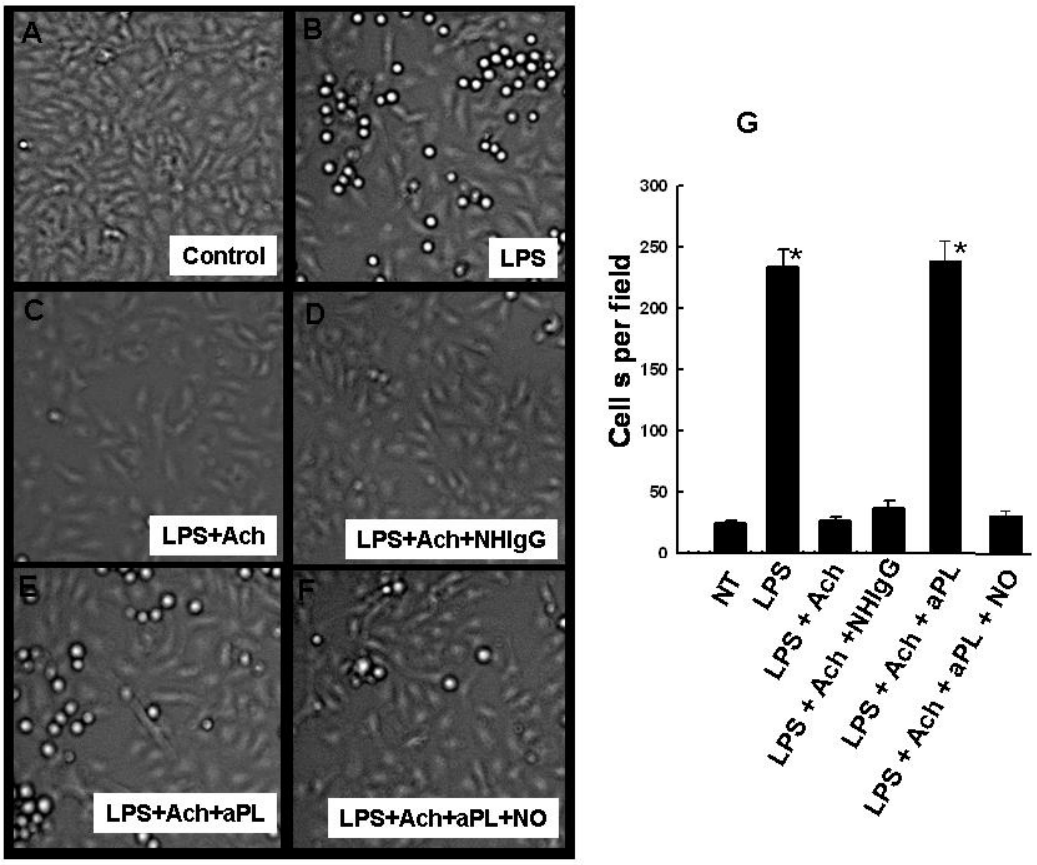

Figure 2-3: aPL- mediated increases in monocyte adhesion are NO-dependent.
Monocyte adhesion to BAEC was evaluated under the following conditions: (**A**) control, (**B**) LPS (100 ng/ml), (**C**) LPS + acetylcholine (Ach, 10μmol/L), (**D**) LPS + Ach + NHIgG (100 μg/ml), (**E**) LPS + Ach + aPL (100 μg/ml), and (**F**) LPS + Ach + aPL + NO donor (S-nitroso-N acetyl-D, L-penicillamine, 20 μmol/L). (**G**) Graph represents summary data. ***** P <0.001 vs. control.

aPL and eNOS activity

The key step to generating NO is the activation of the endothelial nitric oxide synthase (eNOS). To determine if aPL alters eNOS activation, BAEC or

HAEC were pretreated with NHIgG (100μg/ml) or aPL (100μg/ml) and eNOS stimulation by VEGF (100ng/ml) was evaluated. Pretreatment of BAEC with NHIgG had no effect on VEGF-mediated increases in eNOS activation. However, pretreatment with aPL blunted eNOS activation by VEGF (**Figure 2-4A**). Similar results were also obtained with HAEC (**Figure 2-4B**). aPL also blunted acetylcholine-mediated increases in eNOS activation in a mouse endothelial cell line (MFLM-91U) (**Figure 2-4C**). Thus, aPL attenuates eNOS activation by diverse agonists in bovine, human and mouse endothelium.

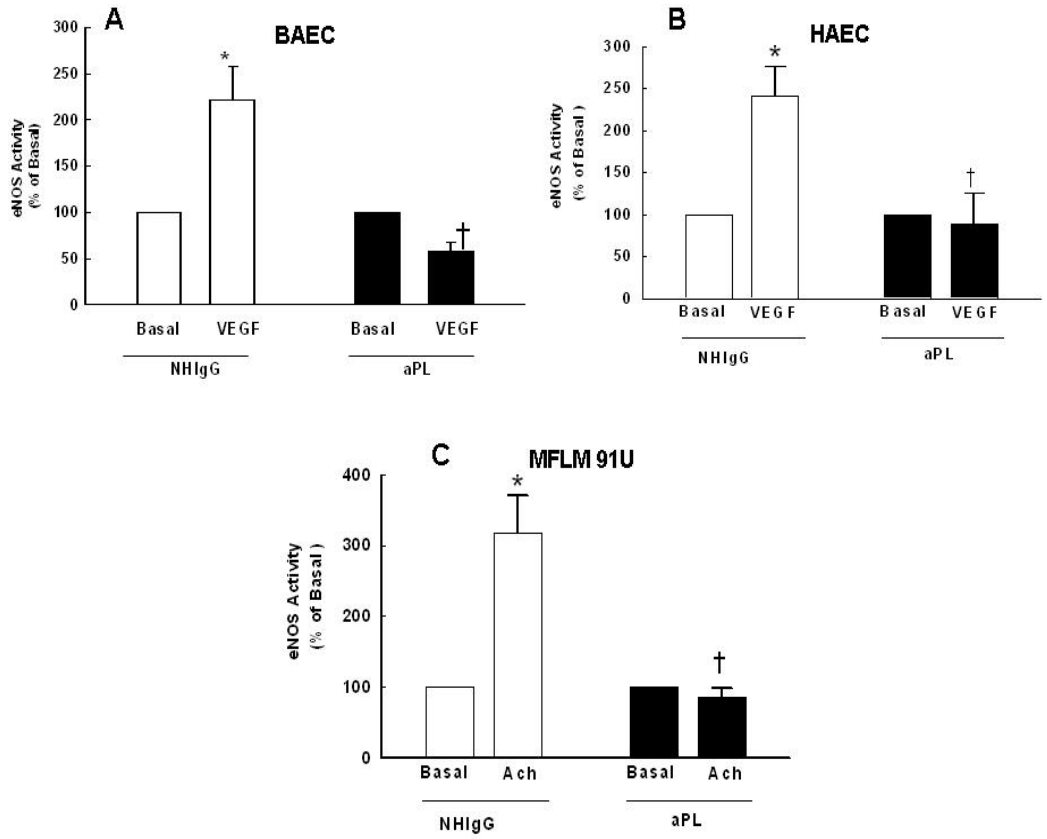

Figure 2-4: aPL antagonizes eNOS activation in cultured endothelial cells. BAEC (**A**) or HAEC (**B**) were pretreated for 15 minutes with NHIgG (100 μg/ml) or aPL (100 μg/ml) and eNOS activity was measured under basal conditions or in the presence of VEGF (100ng/ml) for 15 min. MFLM-91U cells (**C**) were also pretreated with NHIgG or aPL and eNOS activity was measured under basal conditions or in the presence of Ach (10 μmol/L). * $P< 0.05$ vs. basal, † $P< 0.05$ vs. NHIgG.

In order to determine whether aPL attenuates eNOS activation *in vivo*, Ach-mediated changes in carotid artery vascular conductance was measured in C57BL6 mice. At baseline, acetylcholine caused a dose-dependent increase in

vascular conductance in mice (**Figure 2-5A,B ●**). Administration of NHIgG had no effect on Ach-mediated increases in vascular conductance (**Figure 2-5A,** ○). In contrast, aPL administration blunted the Ach response, shifting the dose-response curve downward and to the right (**Figure 2-5B,** ○). Ach-mediated increases in vascular conductance were similarly decreased by the administration of the eNOS antagonist L-NAME following NHIgG or aPL administration (**Figure 2-5A,B ▼**). These findings indicate that the aPL-mediated eNOS antagonism observed in cultured endothelial cells also occurs *in vivo*.

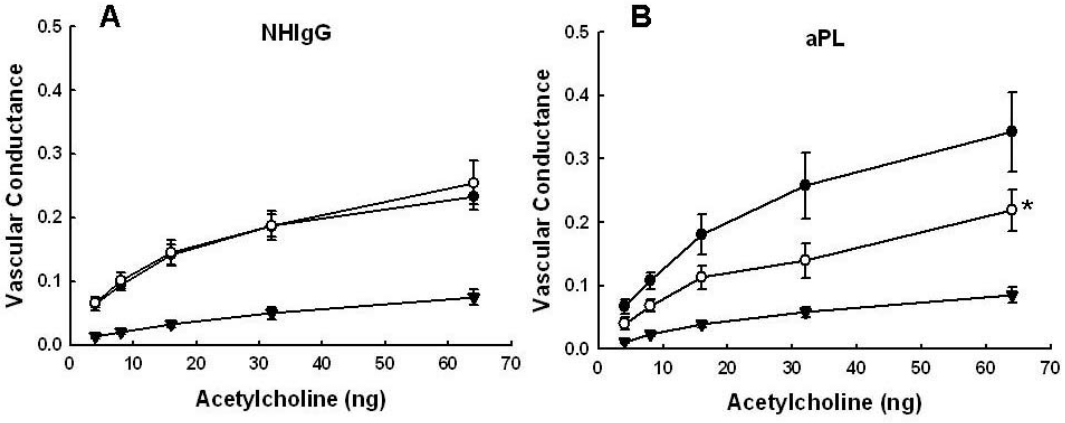

Figure 2-5: aPL antagonizes eNOS activation *in vivo*.
Male C57BL/6 mice were instrumented and changes in carotid vascular conductance in response to eNOS activation were compared following I.V. administration of (A) NH-IgG (2mg) or (**B**) aPL (2mg). Dose responses to Ach were performed sequentially at baseline (control) (●), 60 min after administration of either NHIgG or aPL (○) and 10 minutes after L-NAME administration (▼). Values are mean ± SEM, n=6/ group. * $P<0.05$ vs. control (two-way ANOVA).

Conclusions

APS is characterized by recurrent fetal loss, thrombosis and thrombocytopenia in the presence of circulating aPL. Since the endothelium plays a critical role in thrombosis and inflammation, it has received considerable attention as a target of aPL action. aPL causes endothelial dysfunction and results in a proadhesive and prothrombotic phenotype (Pierangeli et al., 1999; Riboldi et al., 2003). We hypothesized that aPL-mediated endothelial dysfunction and enhanced adhesiveness to monocytes is via declines in NO bioavailability. We further hypothesized that this decrease in NO bioavailability is due to attenuated activity of eNOS.

We have shown that LPS-mediated increases in monocyte adhesion are reversed by activation of eNOS using the agonist, acetylcholine. Blocking the activation of eNOS with L-NAME increases monocyte adhesion to endothelial cells. aPL increases adhesion molecule expression leading to increased adhesion of monocytes to the endothelium (Del Papa et al., 1995; Pierangeli et al., 1999; Riboldi et al., 2003; Simantov et al., 1995). We have demonstrated in our system that aPL increases monocyte adhesion to endothelial cells but NHIgG has no effect. We have also demonstrated that aPL antagonizes the effect of Ach on LPS induced monocyte adhesion, and that this effect can be reversed by an NO donor. Thus, aPL-mediated endothelial cell dysfunction is via decreased NO bioavailibility. Further studies are required to evaluate related changes in

adhesion molecule expression using western blotting or immunofluorescence. Studies also need to be carried out to determine if this process occurs in the endothelium in its native state. This can be tested *ex vivo* with isolated mouse aortas and treatment with aPL and eNOS agonists or antagonists and NO donors. Monocyte adhesion can also be tested *in vivo* in intact mice transfused with fluorescent-labeled monocytes after NHIgG or aPL administration +/- NO donors using intravital microscopy (Cambien et al., 2003; Matsushita et al., 2003).

We have further demonstrated that aPL inhibits the activation of eNOS in cells of human, bovine and mouse origin. These processes are also operative *in vivo* as demonstrated in a mouse model of eNOS activation, where aPL attenuated increases in carotid artery vascular conductance in response to Ach. In these studies we have used a fixed concentration of aPL and NHIgG (100 μg/ml for *in vitro* and 2 mg/mouse for *in vivo*). It would be interesting to test the effect of aPL on eNOS activation using different concentrations of the antibody to determine if there exists a dose-dependent response to aPL. This would help us determine if there is a minimum concentration of aPL required for inhibition of eNOS activation and if there is a dose-dependent inhibition of eNOS activation by aPL. We also need to test the effect of aPL on eNOS activity by other eNOS agonists like estrogen, high density lipoprotein (HDL) or insulin to determine if aPL can inhibit eNOS activation by a wide variety of agonists. Studies of the effect of aPL

on aortic ring relaxation by eNOS agonists can also be carried out (Yuhanna et al., 2001).

Thus, we have demonstrated that aPL-induced declines in NO bioavailability underlie the increased adhesion of monocytes to the endothelium. We have also demonstrated that these declines in NO bioavailability by aPL are due to the attenuation of eNOS activation.

Chapter 3

Nature of aPL Antibodies and Role of Serum Proteins

Introduction

The binding of aPL to yet-to-be identified cell surface proteins and/ or receptors on endothelial cells causes increased expression of adhesion molecules leading to endothelial cell dysfunction. In order to identify putative cell surface receptors and downstream effectors in aPL-mediated eNOS antagonism, we tested the involvement of Fc receptors as candidate receptors for aPL action. Studies have shown that aPL exerts its thrombogenic effects *in vivo* via the crosslinking of Fcγ receptors, which are receptors for IgG, and polymorphisms of the FcγRIIA can be used as a clinical predictor for increased thrombosis risk (Arvieux et al., 1995; Karassa et al., 2003; Sammaritano et al., 1997). However, other studies have been carried out which contradict these results including work indicating that aPL are capable of initiating fetal damage in the absence of activating Fcγ receptors (Atsumi et al., 1998; Girardi et al., 2003). FcγRIIB on the surface of endothelial cells have been implicated in C-reactive protein (CRP) induced inhibition of eNOS activation by different agonists (Mineo et al., 2005).

An antibody consists of four polypeptide chains- 2 heavy chains and 2 light chains joined together to form a "Y" shaped molecule. The amino acid

sequences at the tip of the "Y" vary greatly between different antibodies and are designated the variable region that is responsible for antigen recognition. When the antibody is digested with the enzyme pepsin, the antibody splits into 2 fragments: the Fc fragment containing the constant region of the antibody that bind Fc receptors and the bivalent F(ab')2 fragment (Figure 3-1). To investigate the nature of aPL mediating effects on eNOS, we have tested the region of aPL involved in eNOS inhibition to determine the involvement of Fc receptors using the eNOS activity assay as a readout.

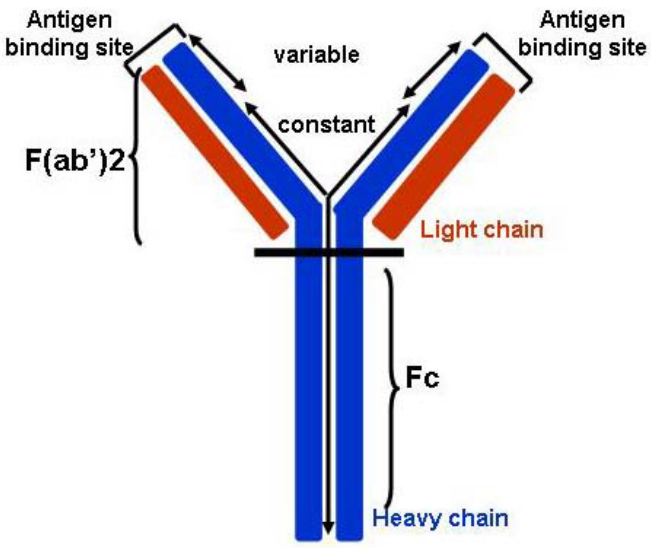

Figure 3-1: Structure of an antibody.
An antibody consists of four polypeptide chains- 2 heavy chains and 2 light chains joined together to form a "Y" shaped molecule. On cleavage by the enzyme pepsin the antibody can be split into 2 fragments: the Fc fragment and the bivalent F(ab')2 fragment containing the variable regions of the antibody.

Regarding serum factors that have been shown to participate in known actions of aPL, components of the complement cascade may be required for aPL-mediated pregnancy loss in mice (Girardi et al., 2001; Holers et al., 2002; Pierangeli et al., 2005; Salmon et al., 2003). We therefore tested the requirement of complement activation in aPL-mediated eNOS inhibition using the eNOS activity assay.

Antibodies purified from APS patients contain a polyclonal mix of antibodies with different specificities. Anti β2GPI antibodies form a major component of the polyclonal aPL antibodies from patients (Galli et al., 1990; McNeil et al., 1990). β2GPI is present in the serum at a concentration of 100-200 μg/ml (Bendixen et al., 1992; Kato et al., 1997; Lozier et al., 1984; Wurm, 1984) and it is present on the surface of endothelial cells. We used serum starvation to test the requirement for serum proteins including β2GPI and also used monoclonal antibodies to β2GPI and different regions of β2GPI to determine its role in aPL-mediated eNOS inhibition.

Methods

Cell Culture: BAEC were cultured as described in *Chapter 1*. For experiments testing the involvement of complement, the cells were cultured in medium containing either 5% FBS or 5% heat-inactivated FBS (56°C waterbath for 30 min which destroys complement) (Triglia and Linscott, 1980). For experiments in which serum-starvation was required, the cells were first cultured in EGM-2 medium with 5% FBS. After the cells reached the desired confluency, they were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) without phenol red and serum for 18 h.

Antibodies used: NHIgG and human polyclonal aPL used were obtained and purified as described in *Chapter 1*. 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 purified by protein G-Sepharose and their purity was assessed by Western blot analysis using antibodies specific for the fragments (Girardi et al., 2003). A β2GPI monoclonal antibody (FC1)-generating hybridoma was kindly provided by Dr. Marc Monestier (Temple University) (Iverson et al., 2002; Monestier et al., 1996). FC1 was purified from culture supernatant by affinity chromatography on recombinant protein G-agarose. Matching IgG1 isotype control mAb was

obtained from Sigma-Aldrich. The mIgG was treated to deplete endotoxin using Detoxi-Gel endotoxin removing gel (Pierce) and tested for purity by the Limulus Amoebocyte Assay (Associates of Cape Cod, Inc.). Additional mouse monoclonal antibodies directed to either domain I or domain II of β2GPI, 3F8 and 2aG4, respectively, were kindly provided by Dr. Philip Thorpe in the Department of Pharmacology at the University of Texas Southwestern Medical Center at Dallas (Luster et al., 2006).

Immunoblot Analysis: BAEC were starved in DMEM lacking serum and phenol red for different time periods and the samples were harvested in SDS sample buffer without 2-mercaptoethanol. Alternatively the cells were serum-starved and reconstituted with purified human β2GPI (Haematologic Technologies Inc.) at 5 μg/ml for 1 hour and then harvested in SDS sample buffer without 2-mercaptoethanol. Results shown were confirmed in 3 independent experiments. Removal of bovine β2GPI and reconstitution with human β2GPI was tested by immunoblotting with monoclonal antibody to domain II of β2GPI (2aG4).

eNOS Activation Assay: eNOS activation was assessed in intact cells by measuring the conversion of [^{14}C]-L-Arginine to [^{14}C]-L- citrulline described in detail in *Chapter 1*. BAEC were preincubated with NHIgG (100 μg/ml), aPL

(100 μg/ml), bivalent F(ab')2 fragment of aPL (100 μg/ml), mIgG (10 μg/ml), FC1 (10 μg/ml), 3F8 (10 μg/ml) or 2aG4 (10 μg/ml) for 15 min, and the incubation with eNOS agonist VEGF (100 ng/ml) was for 15 min.

Statistical Analysis: Data are expressed as mean +/- SEM. Student t-tests or ANOVA was used to assess differences between 2 groups or among more than 2 groups, respectively. Significance was set at $P< 0.05$.

Results

aPL, eNOS Antagonism and Requirement for Fc Receptors

Fc receptors are involved in CRP-mediated antagonism of eNOS (Mineo et al., 2005). In order to determine if Fc receptors are involved in aPL-mediated eNOS antagonism, the requirement of different regions of the aPL antibody for this process was evaluated. eNOS activity was tested in BAEC pretreated with either NHIgG (100μg/ml) or intact aPL (100μg/ml) or the F(ab')2 fragment of aPL (100μg/ml) for 15 min followed by incubation with VEGF for 15 min. The F(ab')2 fragment includes the bivalent antigen recognition variable regions of the antibody but does not include the Fc fragment (**Figure 3-1**). The F(ab')2 fragment of aPL antagonized eNOS activity similar to intact aPL (**Figure 3-2**). Thus, the F(ab')2 fragment of the polyclonal aPL antibodies is sufficient to antagonize eNOS activity and the Fc fragment is not required, indicating that Fc receptors are not the receptor for aPL in endothelium.

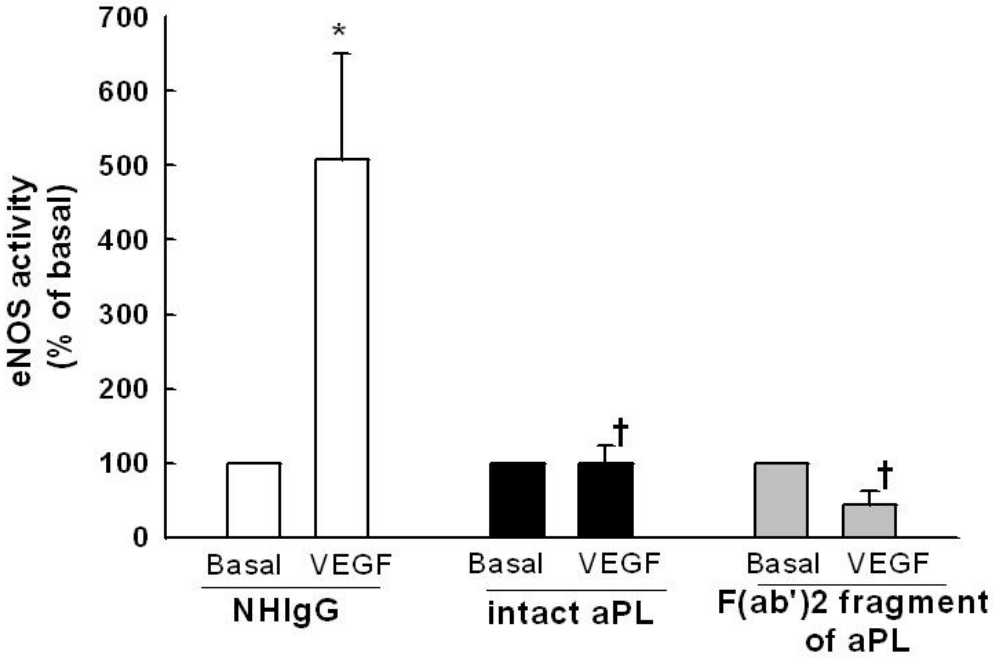

Figure 3-2: aPL-mediated antagonism of eNOS requires the F(ab')2 fragment and is independent of Fc.
BAEC were pretreated for 15 mins with NHIgG (100 μg/ml), intact aPL (100 μg/ml) or F(ab')2 fragment of aPL (100 μg/ml), and eNOS activity was measured under basal conditions or in the presence of VEGF (100ng/ml) for 15 min. ***** P< 0.05 vs. basal, † P< 0.05 vs. NHIgG.

Requirement for Complement

In order to determine if complement is involved in aPL-mediated eNOS antagonism, the effect of culturing endothelial cells in medium containing complement (EGM-2 with regular serum) or complement-free medium (EGM-2 with heat-inactivated serum) was tested using the eNOS activity assay. Cells cultured in either regular serum (**Figure 3-3A**) or heat-inactivated serum (**Figure**

3-3B) were pretreated with aPL (100μg/ml) or NHIgG (100μg/ml) for 15 min and eNOS was stimulated with VEGF for 15 min. aPL-mediated eNOS antagonism was not altered in the presence of heat-inactivated serum, indicating that complement is not required.

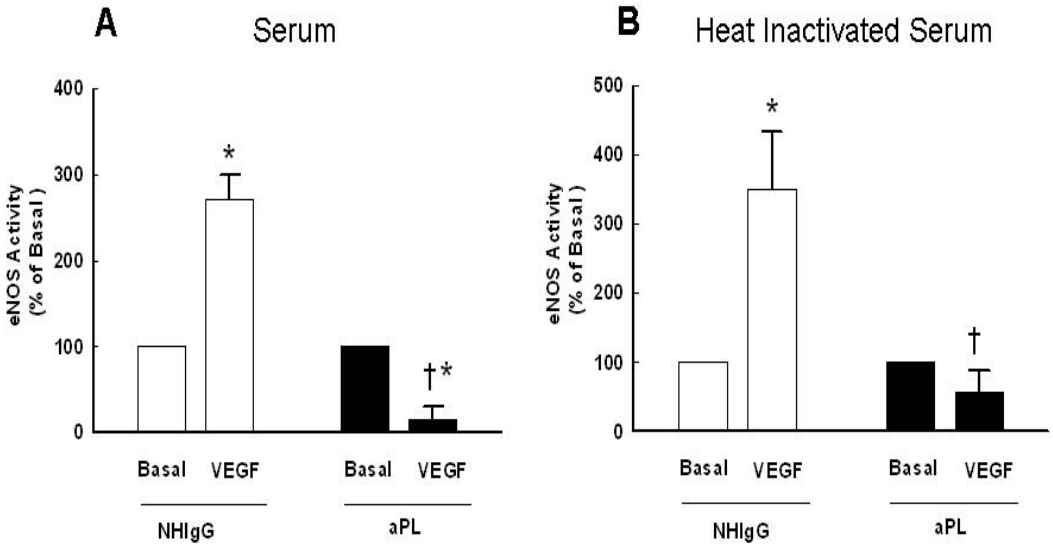

Figure 3-3: aPL-mediated eNOS antagonism is independent of complement. BAEC cultured in media with (**A**) untreated serum or (**B**) heat inactivated, complement-free serum were pretreated for 15 min with NHIgG (100 μg/ml) or aPL (100 μg/ml), and eNOS activity was measured under basal conditions or in the presence of VEGF (100ng/ml) for 15 min. * $P< 0.05$ vs. basal, † $P< 0.05$ vs. NH-IgG.

Requirement for β2GPI

In order to prepare for experiments evaluating mechanisms underlying eNOS antagonism by aPL, specifically phosphorylation/ dephosphorylation events, the effects of aPL were tested under conditions of serum deprivation. Serum deprivation is required to reliably evaluate eNOS phosphorylation or dephosphorylation in cultured endothelial cells. In BAEC cultured in the presence of serum, aPL antagonism of eNOS activity was apparent (**Figure 3-4, left**). However, after the cells were serum deprived for 18 hours, aPL did not inhibit eNOS activity (**Figure 3-4, right**). Thus, a serum component is required for aPL antagonism of eNOS.

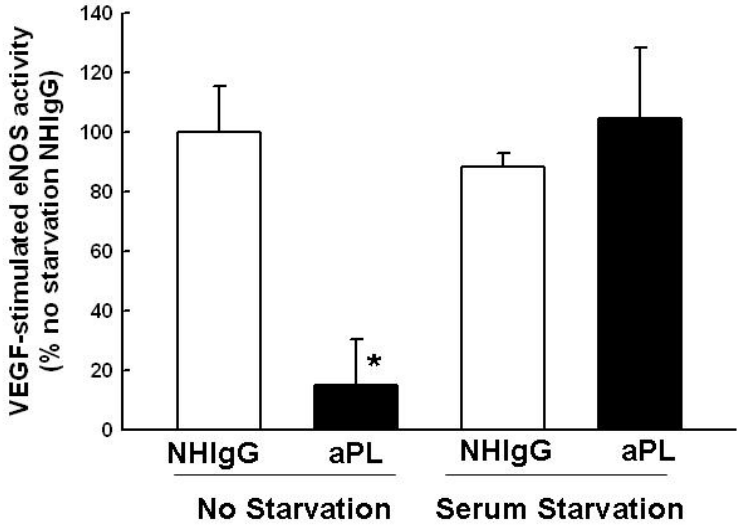

Figure 3-4: aPL antagonism of eNOS requires a serum component.
BAEC cultured in serum-containing media or serum-starved for 18 h were pretreated for 15 min with NHIgG (100 μg/ml) or aPL (100 μg/ml), and eNOS activity was measured under basal conditions or in the presence of VEGF (100ng/ml) over 15 min. * $P < 0.05$ vs. no aPL.

β2GPI is removed from endothelial cells by serum deprivation (Del Papa et al., 1997; Del Papa et al., 1995). The loss of β2GPI from BAEC in our model system was tested by immunoblotting after serum deprivation (**Figure 3-5, left**). To establish β2GPI reconstitution after serum starvation, purified human β2GPI was then added to the cells at a concentration of 5 μg/ml for 1 hour and immunoblotting was performed (**Figure 3-5, right**) (Del Papa et al., 1997; Del Papa et al., 1995). The bovine β2GPI runs at a higher molecular weight than the human isoform of β2GPI as indicated in **Figure 3-5**. The studies showed that

β2GPI is removed from endothelial cells by serum-starvation and it is effectively reconstituted after serum deprivation using purified β2GPI protein.

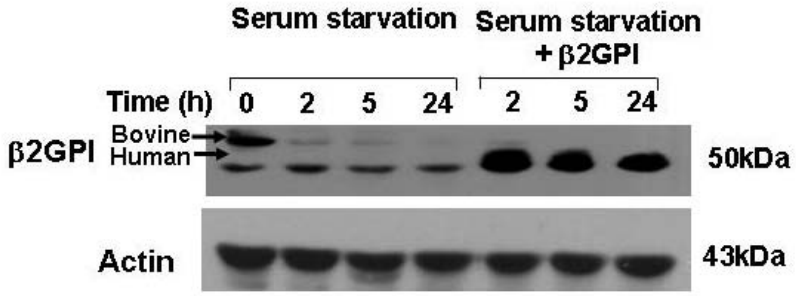

Figure 3-5: Loss of β2GPI from endothelial cell surface after serum starvation and reconstitution with purified human β2GPI.
BAEC were serum starved for 0-24h and harvested, or treated with human β2GPI (5μg/ml) for 1 h after serum starvation for 2-24h. Cell lysates were immunoblotted for β2GPI. Findings shown are representative of 3 independent experiments.

Having confirmed that β2GPI is lost by serum starvation, we then tested if β2GPI is the serum component required for aPL action. To do so the effect of a β2GPI monoclonal antibody on eNOS activity was evaluated. BAEC were pretreated with control IgG (NHIgG, 100μg/ml or mIgG1κ, 10 μg/ml) or FC1 (β2GPI monoclonal antibody, 10 μg/ml) for 15 min and eNOS stimulation by VEGF was evaluated. While the control antibody did not alter eNOS activation,

FC1 inhibited eNOS activation (**Figure 3-6**). These findings indicate that monoclonal antibody to β2GPI causes eNOS antagonism similar to aPL.

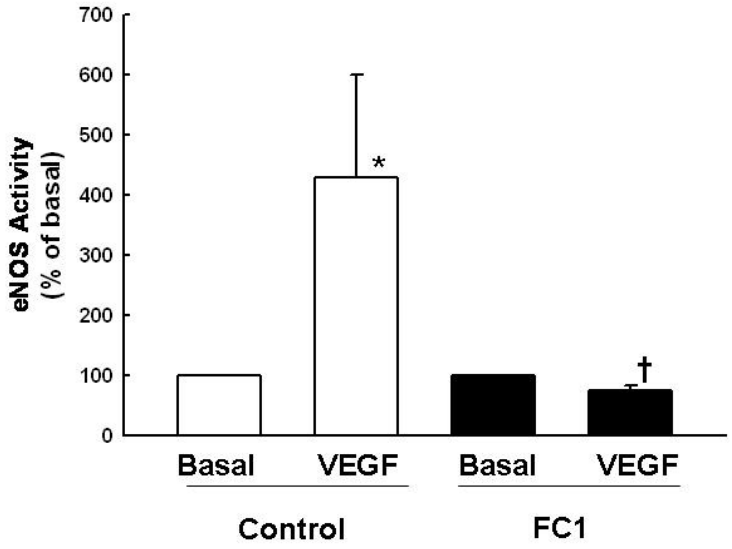

Figure 3-6: Monoclonal antibody to β2GPI (FC1) antagonizes eNOS activity. BAEC were pretreated for 15 min with control IgG or monoclonal antibody to β2GPI (FC1, 10 μg/ml) and eNOS activity was measured under basal conditions or in the presence of VEGF (100ng/ml) for 15 min. * $P< 0.05$ vs. basal, † $P< 0.05$ vs. control.

To determine which domain of β2GPI is important for eNOS antagonism, the effect of monoclonal antibodies to domain I and domain II of β2GPI were tested in the eNOS activity assay. BAEC were pretreated with NHIgG (100 μg/ml), aPL (100 μg/ml), monoclonal antibody to β2GPI domain I (3F8, 10

μg/ml) or monoclonal antibody to β2GPI domain II (2aG4, 10 μg/ml) for 15 min, and treatment with eNOS agonist VEGF was for an additional 15 min. Whereas NHIgG and 2aG4 had no effect on eNOS activation, both aPL and 3F8 attenuate eNOS activation (**Figure 3-7**).

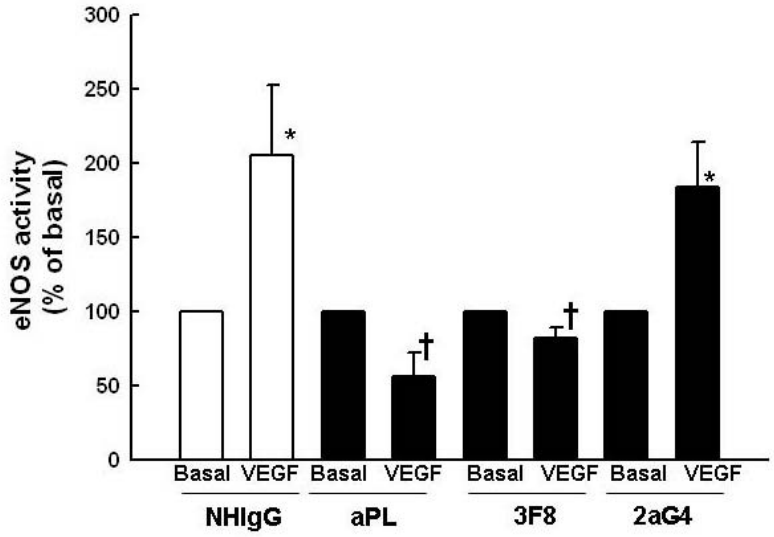

Figure 3-7: Antibody to β2GPI domain I antagonizes eNOS activity.
BAEC were pretreated for 15 min with NHIgG (100μg/ml), aPL (100μg/ml), monoclonal antibody to domain I of β2GPI (3F8, 10 μg/ml) or monoclonal antibody to domain II of β2GPI (2aG4, 10 μg/ml), and NOS activity was measured under basal conditions or in the presence of VEGF (100ng/ml) for 15 min. * $P< 0.05$ vs. basal, † $P< 0.05$ vs. NHIgG.

Thus, monoclonal antibody to β2GPI domain I antagonizes eNOS activation similar to aPL, indicating that β2GPI domain I is important for aPL-mediated eNOS antagonism.

Conclusions

aPL are associated with thrombosis and pregnancy loss in patients with systemic lupus erythematosus (SLE) and APS. aPL are pathogenic *in vivo* as demonstrated by studies in animal models of thrombosis, endothelial cell activation and pregnancy loss. However, the mechanism/s by which aPL mediates these processes is not well understood as knowledge is limited by the polyspecificity of the antibody. In order to further understand the mechanisms underlying aPL-mediated eNOS antagonism, it is important to identify the region of the antibody involved in this inhibition and molecule/s that it recognizes.

We have determined that aPL-mediated eNOS antagonism by polyclonal aPL occurs via its bivalent F(ab')2 fragment and not the Fc fragment of the antibody. This excludes the possibility of involvement of Fc receptors, which are required for CRP-mediated eNOS inhibition (Mineo et al., 2005). A proposed mechanism of aPL action on placenta includes activation of complement leading to generation of potent anaphylatoxins like C5a. Generation of C5a leads to neutrophil and monocyte recruitment and stimulates an immune response. This leads to further activation of the complement cascade causing inflammation in the placenta and fetal injury leading to either fetal growth retardation or fetal death *in utero* (Girardi et al., 2006). However, our results demonstrate that the complement system is not required for aPL-mediated eNOS inhibition. Further evidence to demonstrate that aPL-mediated eNOS inhibition does not require

complement comes from the observation that aPL inhibits eNOS activation in MFLM91U cells (**Chapter 2, Figure 2-4C**), which are cultured in medium with no added serum. Fetal loss in the presence of aPL is from a combination of processes including activation of the complement cascade and increased inflammation and thrombosis in the placental vasculature. It is possible that increased thrombosis occurs via attenuation of eNOS activity by the bivalent F(ab')2 fragment, and that complement activation contributes to placental inflammation.

Although originally thought to bind to anionic phospholipids, it is now widely accepted that aPL recognizes phospholipid-binding proteins including β2GPI (Galli et al., 1990; McNeil et al., 1990). β2GPI is a cationic cell surface protein consisting of five highly conserved domains (I-V). It binds to phospholipids via domain V (Hagihara et al., 1995; Hunt and Krilis, 1994; Hunt et al., 1993; Matsuura et al., 1995). We have demonstrated via serum starvation and using β2GPI monoclonal antibodies that β2GPI is likely a major antigen for aPL-mediated eNOS inhibition. We have also been able to reconstitute β2GPI on endothelial cells after serum starvation using purified human β2GPI. The reconstitution of β2GPI after serum-starvation will be useful in analyzing the effect of aPL on mechanisms of eNOS regulation since serum-starvation is required to reliably observe changes in protein phosphorylation in endothelial cells.

It has been shown using patient samples and β2GPI domain deletion mutants that aPL antibodies recognize an epitope in domain I of β2GPI. Thromboembolic complications are best associated with patient antibodies directed against domain I of β2GPI (de Groot and Derksen, 2005). Antibodies directed against domain I of β2GPI also show a positive β2GPI-dependent lupus anticoagulant (LAC) assay indicating that these antibodies are pathogenic (de Groot and Derksen, 2005). We have shown that domain I of β2GPI, which is recognized by pathogenic aPL, is important for eNOS inhibition, indicating the potential pathological significance of eNOS inhibition.

Further studies need to be carried out using β2GPI monoclonal antibodies *in vivo* to test their effects on eNOS activation. We would expect that monoclonal antibodies to β2GPI (also monoclonal antibody to domain I of β2GPI) would inhibit Ach-mediated increases in carotid artery vascular conductance similar to the polyclonal aPL tested in *Chapter 1*.

Chapter 4

Mechanism of eNOS Inhibition by aPL

Introduction

The endothelial isoform of nitric oxide synthase (eNOS) is the primary source of vascular NO under normal conditions. The NO generated regulates endothelial cell phenotype by various means including down-regulating the expression of adhesion molecules, endothelin-1, cytokines and tissue factor (Boger et al., 1996; Loscalzo, 1995; Wever et al., 1998). The enzymatic activity of eNOS is modulated by phosphorylation of the enzyme and various protein-protein interactions (Fleming and Busse, 1999; Fulton et al., 2001; Kone, 2000; Shaul, 2002; Venema, 2002).

Here we have investigated how aPL impacts mechanisms that regulate eNOS activation, including calcium homeostasis, subcellular localization of the enzyme and eNOS phosphorylation and dephosphorylation. We have tested the effect of aPL on eNOS activation using a calcium ionophore as an eNOS agonist. Attempts were also made to test the effect of aPL on agonist-mediated calcium influx into endothelial cells and on localization of the enzyme. The effect of aPL on agonist-mediated changes in phosphorylation was also investigated. Since we have shown that β2GPI is required for aPL-mediated eNOS inhibition (*Chapter*

3), we tested the effect of aPL on eNOS phosphorylation after serum starvation and after serum-starvation followed by β2GPI reconstitution. We have also tested the effect of aPL on mechanisms involved in the regulation of eNOS phosphorylation.

Methods

Cell Culture: BAEC were cultured as described in *Chapter 1*. For experiments in which serum-starvation was required, the cells were first cultured in EGM-2 medium with 5% FBS. After the cells reached the desired confluency, they were cultured in DMEM without phenol red and serum (DMEM) (Sigma) for 18 h.

Antibodies: NHIgG and aPL were obtained and purified as described in *Chapter 1*. FC1 was obtained and purified as described in *Chapter 3*.

Calcium Measurements: BAEC were cultured on glass coverslips and were loaded with Fura-2AM (Molecular Probes) and $[Ca^{+2}]_i$ was imaged as previously described (Shin et al., 2001). In brief, to measure changes in $[Ca^{+2}]_i$ in response to agonists, the ratio of Fura2 fluorescence measured at excitation wavelengths of 340 and 380 nm and emission wavelength 510 nm was calculated. Fluorescence signal at time 0 (F_0), and at different times (t) after treatment with agonist was measured and the F_t/F_0 ratio (F_t is the fluorescence at time t) was calculated.

Immunoblot Analysis: Immunoblots were performed to assess eNOS phosphorylation using rabbit polyclonal anti-phospho eNOS S1179 antibody (Cell Signaling Technology) and rabbit polyclonal anti-phospho eNOS T495 antibody

(Cell Signaling Technology). Total eNOS abundance was assessed using eNOS monoclonal antibody (BD Biosciences Pharmingen). Immunoblots for assessing Akt phosphorylation were performed using rabbit polyclonal anti-phospho Akt S473 (Cell Signaling Technology) and total Akt abundance using rabbit polyclonal Akt antibody (Cell Signaling Technology). BAEC were starved overnight in DMEM lacking serum and phenol red, reconstituted with purified human β2GPI (Haematologic Technologies Inc.) at 5 μg/ml concentration for 1 hour and then treated with either aPL or NHIgG and eNOS agonist. Results shown were confirmed in 3 independent experiments. Loss of bovine β2GPI with serum starvation and reconstitution of human β2GPI was tested by immunoblotting with antibody to β2GPI domain II (2aG4).

eNOS Activation Assay: eNOS activation was assessed in intact cells by measuring the conversion of [^{14}C]-L-Arginine to [^{14}C]-L- citrulline, described in detail in *Chapter 1*. In some experiments the calcium ionophore ionomycin (Calbiochem) was used to activate eNOS at a final concentration of 1 μM for 15 min.

Statistical Analysis: Data are expressed as mean +/- SEM. Student t-tests or ANOVA was used to assess differences between 2 groups or among more than 2 groups, respectively. Significance was set at $P< 0.05$.

Results

aPL and Calcium Homeostasis in Endothelial Cells

In preparation for studies of the effect of aPL on intracellular calcium, attempts were made to assess intracellular calcium homeostasis in endothelial cells. Endothelial cells cultured on glass coverslips were loaded with Fura-2AM and $[Ca^{+2}]_i$ was imaged as previously described (Shin et al., 2001). These experiments were carried out in the laboratory of Dr. Shmuel Muallem in the Department of Physiology, University of Texas Southwestern Medical Center at Dallas. BAEC cultured on glass coverslips and loaded with Fura-2AM were allowed to stabilize and bradykinin at a final concentration of 10^{-6}M was added to the cells. **Figure 4-1** shows the tracings for the response of seven individual cells within the microscopic field. Only one cell showed an increase in$[Ca^{+2}]_i$ with bradykinin. However all the cells responded to ionomycin at a final concentration of 5 μM. Similar results were obtained with MFLM91U cells and also using other agonists known to cause an increase in calcium influx in endothelial cells including acetylcholine and VEGF (data not shown). Thus, we were not able to observe a consistent increase in calcium influx with agonist and further studies with aPL were not feasible.

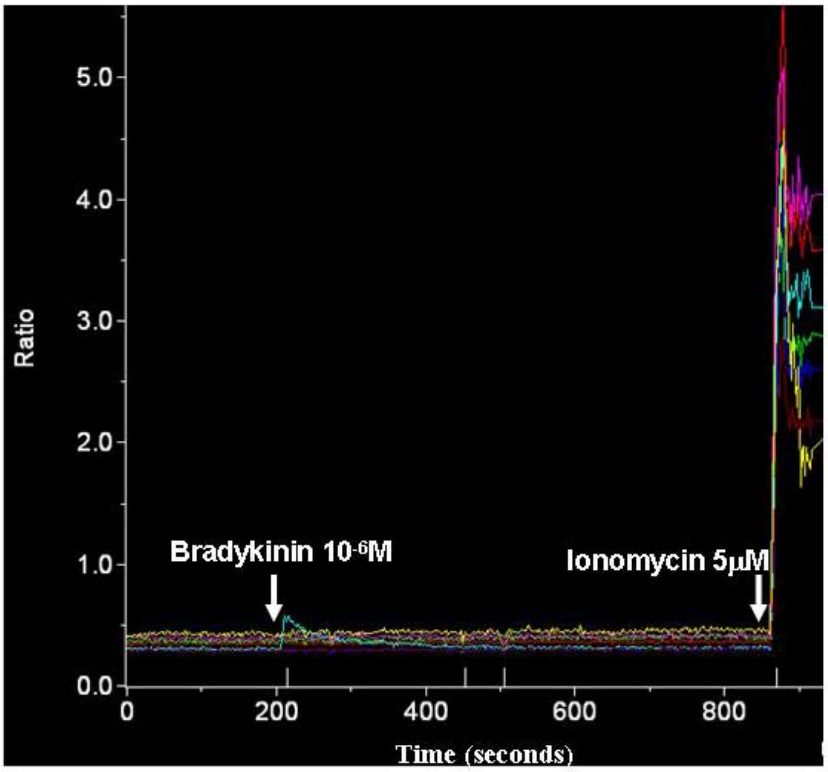

Figure 4-1: Calcium influx in endothelial cells is stimulated by ionomycin but not by bradykinin.
BAEC cultured on coverslips were loaded with Fura-2AM and measurements of calcium induced fluorescence were carried out at excitation wavelengths 340 and 380 nM and the 340/380 ratio calculated. BAEC were treated with 10^{-6}M bradykinin and 5μM ionomycin (indicated by arrows). The 340/380 ratio is represented on the Y axis and the time measured in seconds on the X axis. Each of the traces in the graph represents a single cell.

Calmodulin interacts directly with eNOS leading to an increase in enzyme activity. Attempts were made to co-immunoprecipitate eNOS with calmodulin before and after VEGF treatment of BAEC. However, when the cells are lysed in buffer containing added calcium, the excess calcium causes interaction of eNOS

with calmodulin and in buffer without added calcium calmodulin cannot interact with eNOS. Due to these artifacts caused by the experimental conditions during the assay, our attempts at co-immunoprecipitating eNOS and calmodulin after agonist treatment were unsuccessful (data not shown).

The effect of anti-β2GPI antibody (FC1) on ionomycin-stimulated eNOS activity was determined. The concentration of ionomycin yielding eNOS activation similar to VEGF was determined to be 1μM (data not shown). BAEC were pretreated with control antibody (NHIgG, 100μg/ml) or FC1 (β2GPI monoclonal antibody, 10 μg/ml) for 15 min and eNOS stimulation by ionomycin (1 μM) for 15 min was evaluated. eNOS activation occurred in response to ionomycin in the presence of control IgG, and FC1 partially inhibited eNOS activation by ionomycin (**Figure 4-2**).

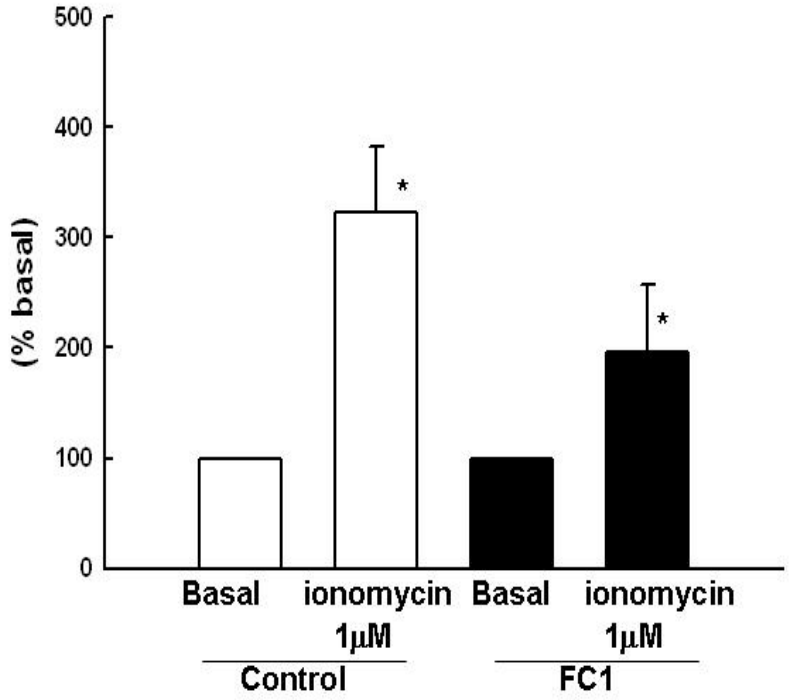

Figure 4-2: Monoclonal antibody to β2GPI (FC1) partially inhibits eNOS activation by ionomycin.
BAEC were pretreated for 15 min with control IgG or monoclonal antibody to β2GPI (FC1, 10 μg/ml) and eNOS activity was measured under basal conditions or in the presence of ionomycin (1μM) for 15 min. ***** P< 0.05 vs. basal.

aPL and eNOS Localization

eNOS is a membrane bound enzyme and membrane localization is important for its activity. We attempted to determine the subcellular localization of eNOS and test the effect of aPL on eNOS localization using immunofluorescence. However, in BAEC we were not able to clearly evaluate

changes in membrane localization of the enzyme. The enzyme was present at the membrane and also at a perinuclear location, most likely in Golgi (data not shown), and under these conditions it was not possible to determine a change in the localization of eNOS after treatment with aPL.

aPL and eNOS Phosphorylation/ Dephosphorylation

The effect of aPL on eNOS S1179 phosphorylation, T497 dephosphorylation and ERK phosphorylation was first determined in the absence of serum. BAEC were cultured in regular medium and serum-starved for 18 h. Cells were then pretreated with NHIgG (100 μg/ml) or aPL (100 μg/ml) for 15 min and for a further 0, 5 or 10 min in the presence of VEGF. Cell lysates were then immunoblotted using respective antibodies to phosphorylated and total eNOS or ERK. In the presence of NHIgG, VEGF caused an increase in eNOS S1179 phosphorylation (**Figure 4-3A**). In the presence of aPL, VEGF-mediated increases in eNOS S1179 phosphorylation were also observed (**Figure4-3A**). VEGF-mediated decreases in T497 dephosphorylation were also not altered in the presence of aPL (**Figure 4-3B**). In addition, increases in ERK phosphorylation by VEGF were also not affected by the presence of aPL (**Figure 4-3C**). Importantly, the studies of the effect of aPL on eNOS phosphorylation after serum starvation were carried out before the requirement for β2GPI was determined (*Chapter 3*). These findings for phosphorylation in the absence of serum are consistent with the

lack of aPL antagonism of eNOS activity under these conditions, confirming the requirement for β2GPI for aPL action.

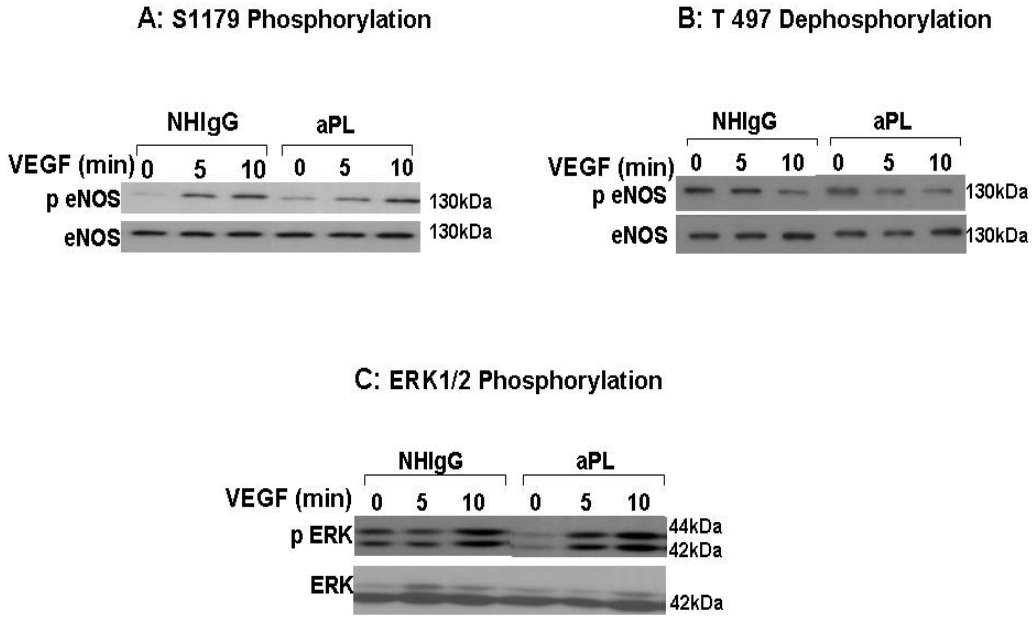

Figure 4-3: aPL does not alter eNOS S1179 phosphorylation, T497 dephosphorylation or p42/44 MAPK phosphorylation after serum starvation. BAEC were serum-starved for 18 h, pretreated for 15 min with NHIgG (100 μg/ml) or aPL (100 μg/ml) and incubated with VEGF (100ng/ml). Lysates were obtained and immunoblotted for (**A**) peNOS and eNOS using polyclonal phospho S1179 eNOS and monoclonal eNOS antibodies, respectively; (**B**) peNOS and eNOS using polyclonal phospho T497 eNOS and monoclonal eNOS antibodies, respectively, and (**C**) pERK1/2 and ERK1/2 using polyclonal phospho ERK1/2 and monoclonal ERK1/2 antibodies, respectively. Findings shown are representative of 3 independent experiments.

The effect of aPL on eNOS S1179 phosphorylation, T497 dephosphorylation and ERK phosphorylation was then determined after serum-starvation and β2GPI reconstitution. BAEC were cultured in regular medium, serum-starved for 18 h and incubated with β2GPI (5 μg/ml, 1 hour). Cells were then pretreated with NHIgG (100 μg/ml) or aPL (100 μg/ml) for 15 min and for a further 0, 5 or 10 min in the presence of VEGF. In the presence of NHIgG, VEGF caused an increase in eNOS S1179 phosphorylation (**Figure 4-4A**). In contrast, after β2GPI reconstitution and in the presence of aPL, the VEGF-mediated increase in eNOS S1179 phosphorylation was blunted (**Figure 4-4A**). Cumulative results for n=8 are depicted graphically in **Figure 4-4B**. The VEGF-mediated decrease in T497 depohsphorylation after β2GPI reconstitution was not altered in the presence of aPL (**Figure 4-4C**). The increase in ERK phosphorylation by VEGF was also not affected by aPL (**Figure 4-4D**). Thus, in the presence of β2GPI, aPL blunts the agonist-mediated increase in eNOS S1179 phosphorylation.

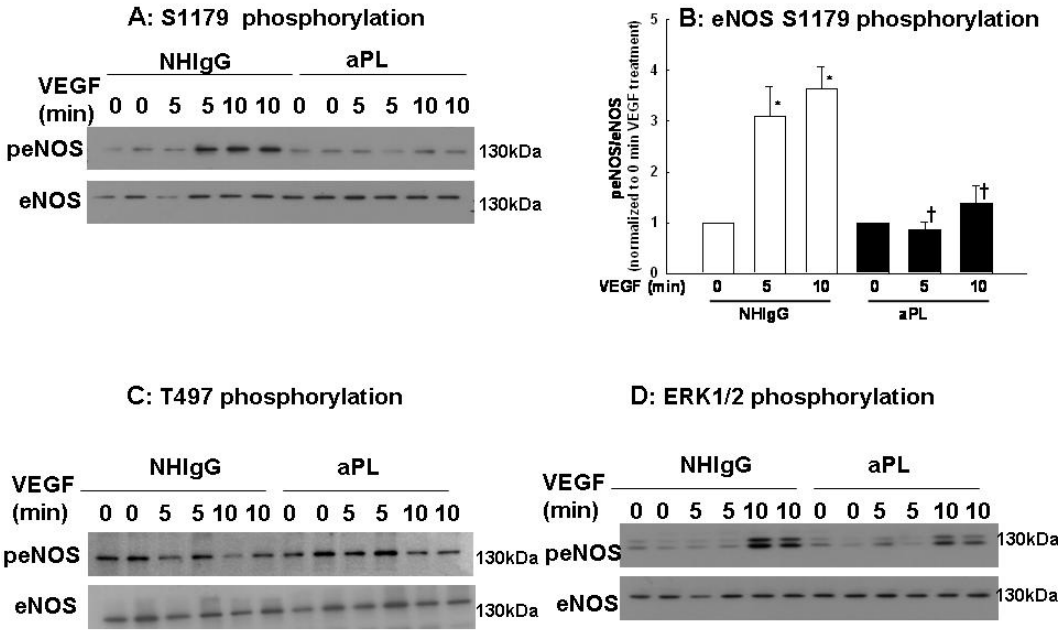

Figure 4-4: aPL inhibits VEGF-mediated eNOS S1179 phosphorylation, but has no effect on T497 dephosphorylation or ERK phosphorylation following serum starvation and β2GPI reconstitution.

BAEC, serum-starved for 18 h and then incubated with β2GPI (5μg/ml) for 1 h, were pretreated for 15 min with NHIgG (100 μg/ml) or aPL (100 μg/ml) and incubated with VEGF (100ng/ml). Lysates were obtained and immunoblotted for (**A**) peNOS and eNOS using polyclonal phospho S1179 eNOS and monoclonal eNOS antibodies, respectively; (**B**) represents the summary data for n=8 from (**A**). * $p<0.05$ vs 0 min of VEGF treatment, † $p<0.05$ vs NHIgG treatment. (**C**) peNOS and eNOS using polyclonal phospho T497 eNOS and monoclonal eNOS antibodies, respectively, and (**D**) pERK1/2 and ERK1/2 using polyclonal phospho ERK1/2 and monoclonal ERK1/2 antibodies, respectively. Findings shown in (**C**) and (**D**) are representative of 3 independent experiments.

The kinase responsible for eNOS S1179 phosphorylation is Akt. In order to determine if aPL alters Akt phosphorylation and activation, the effect of aPL on Akt S473 phosphorylation was determined. BAEC were cultured in regular medium, serum-starved for 18 h and incubated with β2GPI (5 μg/ml, 1 hour). Cells were then pretreated with NHIgG (100 μg/ml) or aPL (100 μg/ml) for 15 min and for a further 10 min in the presence of VEGF. Cell lysates were then immunoblotted using pAkt S473 antibody. Total Akt immunoblot was used as a loading control. In the presence of NHIgG, VEGF caused an increase in Akt S473 phosphorylation (**Figure 4-5**). Similarly, in the presence of aPL, the VEGF-mediated increases in Akt S473 phosphorylation was unchanged (**Figure 4-5**). Thus, aPL does not alter Akt phosphorylation and activation.

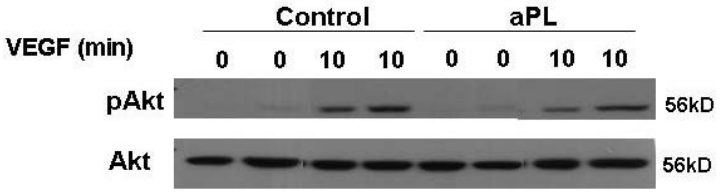

Figure 4-5: aPL does not alter VEGF-mediated Akt phosphorylation.
BAEC serum-starved for 18 h and then incubated with β2GPI (5μg/ml) for 1 h were pretreated for 15 min with NH-IgG (100 μg/ml) or aPL (100 μg/ml) and incubated with VEGF (100ng/ml). Lysates were obtained and immunoblotted for pAkt and total Akt using polyclonal phospho S473 Akt and polyclonal Akt antibodies, respectively. Findings shown are representative of 3 independent experiments.

Conclusions

NO is a key determinant of vascular health that regulates various physiological processes including thrombosis, endothelial-monocyte interaction, vascular cell migration and proliferation and vascular tone and permeability. eNOS is the enzyme responsible for NO production in the endothelium. eNOS is regulated by 3 main mechanisms: changes in $[Ca^{+}2]_i$, subcellular localization of the enzyme and the phosphorylation status of the enzyme.

We attempted to study the effect of aPL on calcium homeostasis in endothelial cells. In order to carry out these experiments, we first attempted to study the effect of different agonists on calcium influx in endothelial cells. Though similar experiments have been performed successfully by different groups using cells in suspension (Lorkowska et al., 2004; Nakajima et al., 2000), our attempts were unsuccessful using cells grown on coverslips and agonists known to cause increase in cytosolic calcium. The cells used, however, responded to ionomycin with an increase in intracellular calcium, indicating that the cells were healthy and responded to strong stimuli. Further experiments using endothelial cell in suspension are needed to determine if aPL alters endothelial cell calcium homeostasis.

To study eNOS-calmodulin interaction in endothelial cells we attempted to co-immunoprecipitate eNOS and calmodulin before and after VEGF treatment

in BAEC. However, as described in the *Results* section, due to experimental conditions required for co-immunoprecipitation these attempts were unsuccessful.

We have demonstrated that aPL only partially inhibits ionomycin-mediated increases in eNOS activity. This indicates that increasing calcium concentration in the cell through non-receptor mediated mechanisms partially overcomes the effects of aPL on eNOS activity. The phosphorylation of eNOS S1179 facilitates increased eNOS-calmodulin interaction leading to increased enzyme activity. However, increases in intracellular $[Ca^{+}2]_i$ can increase eNOS-calmodulin interaction in the absence of agonist-induced eNOS S1179 phosphorylation (Lin et al., 2003; McCabe et al., 2000). Thus, the increased calcium influx into cells with ionomycin partially overcomes the inhibitory effect of non-phosphorylated S1179.

Localization of eNOS to the plasmalemmal caveolae membrane is also important for its activity (Shaul, 2002). Using immunofluorescence, we were unable to detect specific localization of eNOS to the membrane in BAEC. The membrane localization of eNOS in pulmonary artery endothelial cells (PAEC) has been previously demonstrated in our lab using immunofluorescence (Blair et al., 1999). In our immunofluorescence studies using BAEC, we observe eNOS localization both at the membrane and also in a peri-nuclear location that is likely the Golgi. eNOS localization to both the Golgi compartment and the plasma membrane has been shown by other groups (Fulton et al., 2002). Using these

cells it would be difficult to determine change in eNOS localization after aPL treatment. Further studies using cell fractionation techniques are needed to determine if aPL alters the subcellular localization of eNOS.

Phosphorylation/ dephosphorylation of eNOS plays a major role in regulating eNOS activity. Our results from *Chapter 3* determined that aPL-mediated eNOS antagonism requires β2GPI from the serum and that the action of aPL on eNOS activity is lost after serum-starvation. We had also shown using immunoblotting that β2GPI is stripped off BAEC after serum-starvation and can be reconstituted with human β2GPI. Applying these results to study the effect of aPL on eNOS phosphorylation after β2GPI reconstitution, we observed an attenuation of eNOS S1179 phosphorylation by VEGF. This attenuation of eNOS S1179 phosphorylation was not observed in BAEC not reconstituted with β2GPI. These results reinforce the requirement for β2GPI for aPL action on eNOS activity. aPL has no effect on eNOS T497 dephosphorylation with or without b2GPI reconstitution. ERK activation is required for eNOS activation by some agonists like HDL (Mineo and Shaul, 2003; Mineo et al., 2003). ERK activation is not affected by aPL in the presence or absence of β2GPI.

Akt is the kinase responsible for phosphorylation of eNOS by different agonists including VEGF (Fulton et al., 1999). Akt phosphorylation, however was not altered by aPL. Further studies are needed to determine the mechanism

underlying aPL-mediated decreases in eNOS S1179 phosphorylation. Studies need to be carried out to test the involvement of the phosphatase PP2A that is responsible for eNOS dephosphorylation, as it is possible that aPL increases the activity of the phosphatase leading to increased eNOS S1179 dephosphorylation and decreased eNOS activity. This can be done using inhibitors to the phosphatase (okadaic acid) in both eNOS activity assays and immunoblots evaluating eNOS phosphorylation. If studies using pharmacological inhibitors indicate that aPL activates PP2A thus leading to eNOS inhibition, then siRNA of PP2A can be carried out and the effect of aPL tested in this system using eNOS activity and eNOS phosphorylation as endpoints. Expression of constitutively active form of Akt (CA-Akt) in endothelial cells causes eNOS to be phosphorylated in the absence of agonist and to have higher basal enzyme activity. If aPL inhibits eNOS in cells overexpressing CA-Akt, it provides additional evidence that the phosphatase PP2A may be activated to dephosphorylate eNOS. Using these strategies, we can determine the mechanism responsible for aPL-mediated eNOS S1179 dephosphorylation.

Chapter 5

Involvement of LDL Receptor Family Members in aPL-mediated eNOS Antagonism

Introduction

APS is a non-inflammatory autoimmune disease characterized by pregnancy loss and/ or arterial and venous thrombosis in the presence of autoantibodies that primarily recognize β2GPI. It has been previously demonstrated that dimerization of β2GPI causes binding to platelet receptor apolipoprotein E receptor 2' (apoER2') leading to platelet activation (Lutters et al., 2003; van Lummel et al., 2005). apoER2' is a splice variant of apoER2, which is a member of the low density lipoprotein receptor (LDLR) family. Also, anti-β2GPI antibody induced β2GPI dimerization promotes interaction of β2GPI with members of the LDLR family including apoER2, VLDLR, LRP and megalin (Pennings et al., 2006).

Members of the LDL receptor family have been identified in different mammalian cell types. apoER2, a member of this family, is a signal transducer for the secreted protein reelin. Reelin regulates neuronal positioning in the developing brain (May et al., 2005; Schneider and Nimpf, 2003). Although apoER2 is predominantly expressed in the brain, several splice variants of the

protein are also expressed in different tissues (May et al., 2005; Schneider and Nimpf, 2003). Expression of apoER2 and VLDLR in the endothelium has been reported (Korschineck et al., 2001; Sacre et al., 2003; Wyne et al., 1996).

Since aPL-mediated eNOS antagonism requires β2GPI (*Chapter 3*), and apoER2' mediates aPL action in platelets via β2GPI, we tested the involvement of LDLR in eNOS antagonism by aPL. The role of the LDL receptor family was tested using an inhibitor to the LDL receptor family, receptor associated protein (RAP), which directly interacts and inhibits binding of other ligands to members of the LDL receptor family (Herz, 2006). The role of individual members of the LDLR family has also been analysed using siRNA techniques. In addition, studies of aPL and changes in carotid vascular conductance responses to Ach have been carried out in apoER2$^{+/+}$ vs apoER2$^{-/-}$ mice.

Methods

Cell Culture: BAEC were cultured as described in *Chapter 1* and maintained in EGM-2 medium with 5% fetal bovine serum (FBS).

Preparation of GST-RAP and GST proteins: GST tagged Receptor Associated Protein (GST-RAP) and GST control expressing E.coli were kindly provided by Dr. Joachim Herz at University of Texas Southwestern Medical Center at Dallas. GST-RAP and GST were purified as previously described (Herz et al., 1991). The E.coli were cultured in LB medium containing ampicillin (0.1 mg/ml) and incubated at 30 °C for 3-4 h. GST or GST-RAP protein production was induced by addition of 1% isopropyl-beta-D-thiogalactopyranoside (IPTG), the cells were incubated for 5-6 h at 30 °C under shaking conditions, pelleted by centrifugation at 2500 x g for 20 min, resuspended in a buffer containing 15% sucrose, 50mM EDTA, 50mM Tris pH 8.0, protease inhibitors, incubated with lysozyme (10 mg/ml), and lysed using 5% Triton X-100 and mechanical disruption using a 22 ½ gauge needle on ice. The resulting lysate was centrifuged at 20,000 x g for 40 min and the supernatant was used for further purification. Supernatant was combined with glutathione agarose (SIGMA) that was swollen and washed in a buffer containing 10mM Tris, 150mM NaCl, 1mM DTT, pH 7.5, and placed on a rotating shaker overnight at 4 °C. The glutathione beads were collected by

centrifugation and washed 2-3 times in a buffer containing 10mM Tris, 150mM NaCl, 1mM DTT, pH 7.5. The beads were then transferred to a chromatography column and the column eluted with 25mM glutathione in the same buffer. 1 ml fractions of the eluate were collected and the fractions containing protein were collected and pooled. The pooled fractions were then dialyzed at 4 °C using a buffer containing 10mM Tris, 150mM NaCl, pH 7.5 using Slide-A- Lyzer® dialysis cassettes (Pierce). The dialyzed protein fraction was aliquoted and stored at -80°C. Purity of the protein was assessed by SDS-PAGE and coomassie staining.

siRNA Preparation and Transfection: Double-stranded (ds) RNA were designed to target the open reading frame of the bovine isoforms of ApoER2, VLDLR and Annexin A2 (Mineo et al., 2005; Seetharam et al., 2006) with sequences listed in the table below. A dsRNA (control siRNA, sequence in **Table 1** below) served as control.

Table 1

Target Protein	Target sequence
Bovine ApoER2	5'- ACUGGAAGCGGAAGAAUAC -3'
Bovine VLDLR	5'- AGAAUGCCAUGUAAAUGAA -3'
dsRNA control RNA	5'-AGUUAGACCAGACCGAGGATT-3'

BAEC were transfected with 40 nM RNA according to the procedures described previously (Mineo et al., 2005; Seetharam et al., 2006). 24 h after transfection, eNOS activity studies were performed in the presence or absence of aPL or control antibodies. The knockdown of the proteins was confirmed by immunoblot analysis.

Immunoblot Analysis: Immunoblots were performed to assess the efficiency of siRNA targeting of apoER2 and VLDLR using rabbit polyclonal antibodies kindly provided by Dr. Joachim Herz in the Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas. BAEC transfected with either apoER2, VLDLR or both the dsRNAs, or control dsRNA (control) were harvested 24 hours post-transfection in SDS sample buffer. Actin was quantitated by immunoblotting using goat actin polyclonal antibody (SantaCruz Biotechnology Inc.).

eNOS Activity Assay: eNOS activation was assessed in intact cells by measuring the conversion of [^{14}C]-L-Arginine to [^{14}C]-L- citrulline, described in detail in *Chapter 1*. BAEC were preincubated with NHIgG (100 μg/ml) or aPL (100 μg/ml) in the presence of GST (12 μg/ml) or GST-RAP (30 μg/ml) for 15 min and incubation with VEGF (100 ng/ml) was for 15 min. Assays to test the activity of eNOS after control siRNA or apoER2 or VLDLR or the double siRNA of apoER2 and VLDLR were also carried out after pretreatment with NHIgG (100 μg/ml) or aPL (100 μg/ml) for 15 min and incubation with VEGF for an additional 15 min.

Carotid Artery Vascular Conductance in mice: To determine if aPL alters eNOS activity in apoER2$^{+/+}$ vs. apoER2$^{-/-}$ mice, Ach-mediated increases in carotid vascular conductance were measured in these mice before and after aPL (2mg) or NH-IgG (2mg) administration as described in detail in *Chapter 1*. The mice used were between 12-16 weeks of age and were provided by Dr. Joachim Herz from the Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas.

Statistical Analysis: Data are expressed as mean +/- SEM. Student t-tests or ANOVA was used to assess differences between 2 groups or among more than 2 groups respectively. Significance was set at $P< 0.05$.

Results

aPL-mediated eNOS Antagonism and LDLR family

In order to determine if one/ more members of the LDLR family are involved in aPL-mediated eNOS inhibition, the effect of aPL on eNOS activity was tested in the presence of an inhibitor of the LDL receptor family, receptor associated protein (RAP). BAEC were pretreated with NHIgG (100 μg/ml) or aPL (100 μg/ml) in the presence of either GST control (12 μg/ml) or RAP-GST (30 μg/ml) for 15 min followed by treatment with VEGF for an additional 15 min. The presence of GST control did not alter aPL-mediated eNOS antagonism (**Figure 5-1, left**). However, in the presence of RAP-GST, aPL did not inhibit eNOS activity (**Figure 5-1, right**). Thus, one/ more members of the LDLR family of proteins are required for aPL- mediated eNOS antagonism.

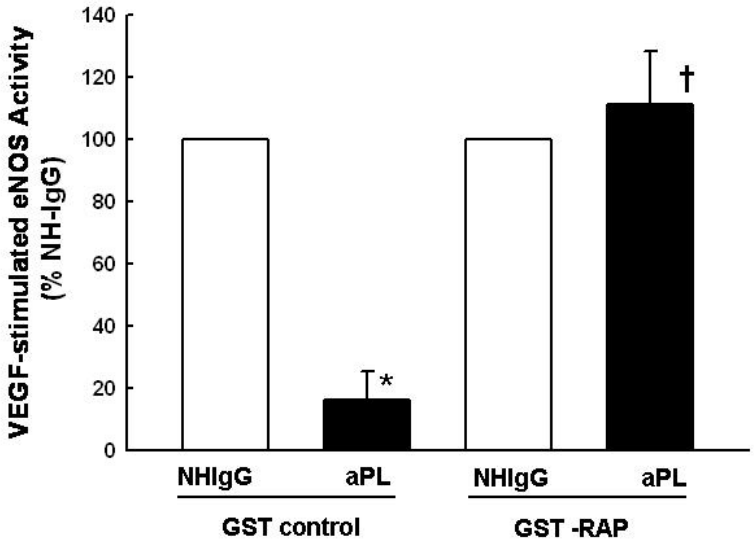

Figure 5-1: RAP prevents aPL-mediated eNOS antagonism.
BAEC were pretreated for 15 min with GST (12 μg/ml) or GST-RAP (30 μg/ml) and either NHIgG (100 μg/ml) or aPL (100 μg/ml), and eNOS activity was measured under basal conditions or in the presence of VEGF (100ng/ml) for 15 min. * $p<0.05$ vs. NHIgG, † $p<0.05$ vs. GST.

Studies in platelets have shown that apoER2' mediates aPL action via β2GPI (Lutters et al., 2003; van Lummel et al., 2005). Results from *Chapter 3* demonstrate the requirement of β2GPI for aPL-mediated eNOS antagonism. On the basis of these two observations, the effect of apoER2 siRNA on aPL-mediated eNOS inhibition was studied. siRNA of VLDLR that can compensate for apoER2

in the neurons and double siRNA of apoER2 and VLDLR were also performed in endothelial cells.

apoER2 siRNA

BAEC were transfected with control dsRNA or apoER2 dsRNA and cells were harvested 24 hours after transfection. Cell lysates were immunoblotted for apoER2 protein using a polyclonal antibody to apoER2. There was a loss of signal for apoER2 protein in cells transfected with apoER2 dsRNA compared to control transfected cells (**Figure 5-2A**). In order to determine if knockdown of apoER2 protein alters aPL-mediated eNOS antagonism, the effect of aPL on eNOS activity was tested in cells transfected with either control siRNA or apoER2 siRNA. BAEC were transfected with dsRNA and 24h post-transfection the cells were pretreated with NHIgG (100 μg/ml) or aPL (100 μg/ml) for 15 min and incubation with VEGF was for an additional 15 min. In the presence of control siRNA, aPL inhibited eNOS activation (**Figure 5-2B**). Similarly, after knockdown of apoER2 by siRNA, aPL antagonized eNOS activation by VEGF (**Figure 5-2B**). Thus, loss of apoER2 by siRNA did not alter aPL-mediated eNOS antagonism.

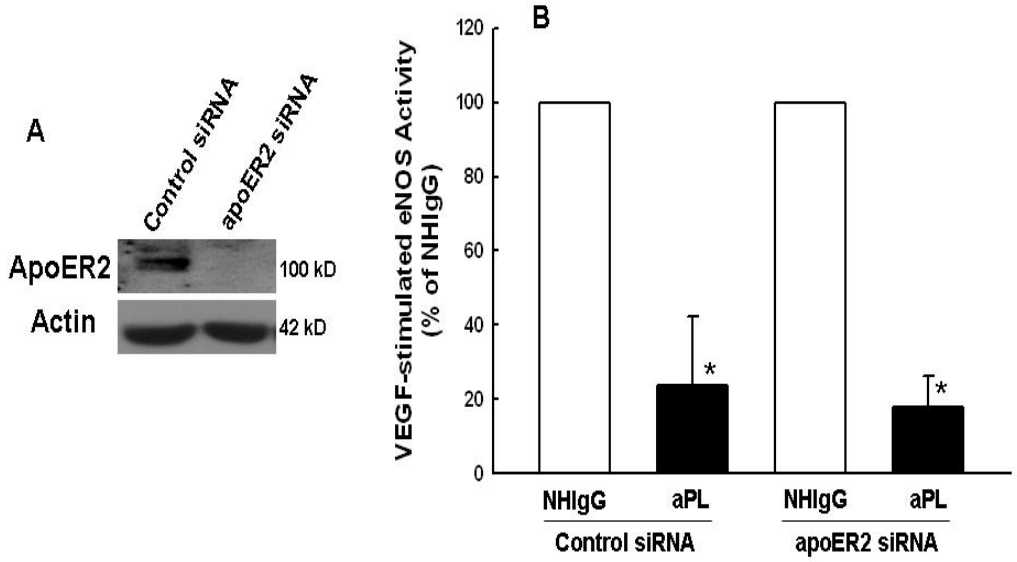

Figure 5-2: Loss of apoER2 by siRNA does not alter aPL-mediated eNOS antagonism.
(**A**) BAEC were transfected with scrambled (control) or apoER2 siRNA and 24 h later apoER2 protein expression was assessed by immunoblotting. (**B**) BAEC were transfected with scrambled (control) or apoER2 siRNA and 24 h later eNOS activation in response to VEGF (100ng/ml, 15 min) was assessed following pretreatment for 15 min with NHIgG (100μg/ml) or aPL(100μg/ml). ***** P<0.05 vs. NH-IgG.

VLDLR siRNA

BAEC were transfected with control dsRNA or VLDLR dsRNA and cells were harvested 24 hours after transfection. Cell lysates were immunoblotted for VLDLR protein using a polyclonal antibody to VLDLR. There was approximately an 80% decrease in the VLDLR protein expression in cells transfected with VLDLR dsRNA compared to control transfected cells (**Figure 5-**

3A). Expression of apoER2 was not altered by the VLDLR siRNA (**Figure 5-3A**). In order to determine if knockdown of VLDLR protein alters aPL-mediated eNOS antagonism, the effect of aPL on eNOS activity was tested in cells transfected with either control siRNA or VLDLR siRNA. BAEC were transfected with dsRNA and 24h post-transfection, the cells were pretreated with NHIgG (100 μg/ml) or aPL (100 μg/ml) for 15 min and incubation with VEGF was for an additional 15 min. In the presence of control siRNA, aPL inhibited eNOS activation (**Figure 5-3B**). Similarly, after knockdown of VLDLR by siRNA, aPL antagonized eNOS activation by VEGF (**Figure 5-3B**). Thus, loss of VLDLR by siRNA has no effect on aPL-mediated eNOS antagonism.

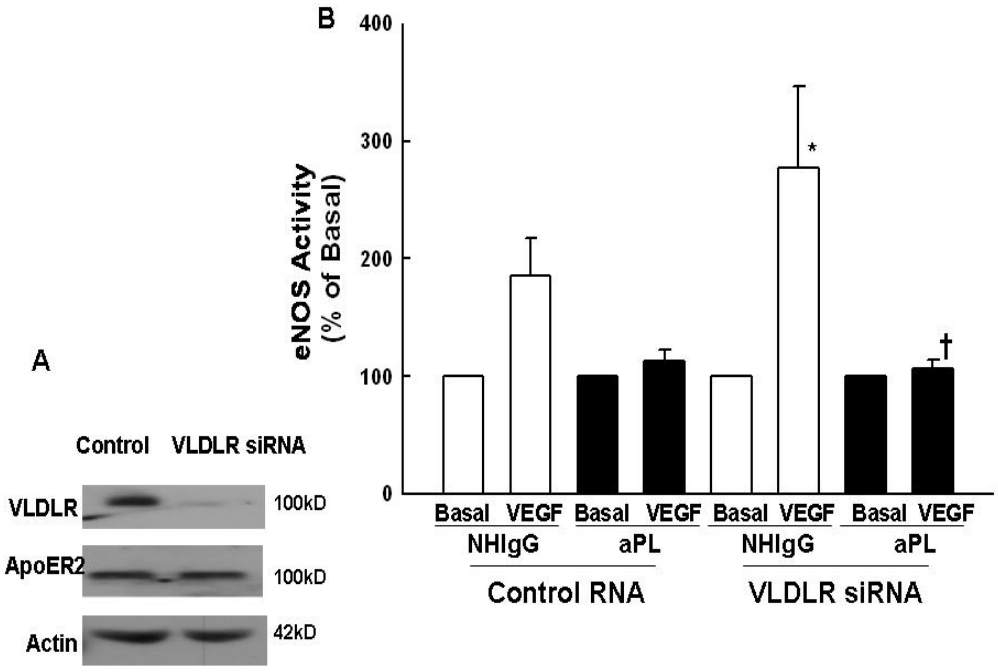

Figure 5-3: Loss of VLDLR by siRNA does not alter aPL-mediated eNOS antagonism.
(**A**) BAEC were transfected with scrambled (control) or VLDLR siRNA and 24 h later VLDLR and apoER2 protein expression was assessed by immunoblotting. (**B**) BAEC were transfected with scrambled (control) or VLDLR siRNA and 24 h later eNOS activation in response to VEGF (100ng/ml, 15 min) was assessed following pretreatment for 15 min with NHIgG (100μg/ml) or aPL(100μg/ml).
***** P<0.05 vs. basal.

apoER2/ VLDLR double siRNA

apoER2 and VLDLR act as signal transducers and receptors for the secreted glycoprotein reelin, which regulates neuronal positioning in laminated structures of the developing brain (May et al., 2005). Although they are members of the

LDLR family, inactivation of either apoER2 or VLDLR does not result in disruptions in lipid homeostasis. However, the double knockout mice display a neurodevelopment defect similar to reelin knockout mice (reeler mouse) (May et al., 2005; Trommsdorff et al., 1999). Since, apoER2 and VLDLR can compensate for each other in neurons, it is possible that they can compensate for each other in endothelial cells. The effect of knockdown of both apoER2 and VLDLR was tested in endothelial cells. BAEC were transfected with control dsRNA or both apoER2 and VLDLR dsRNA and cells were harvested 24 hours after transfection. Cell lysates were immunoblotted for apoER2 and VLDLR proteins. There was minimal to no detection of the apoER2 and VLDLR in cells transfected with apoER2 and VLDLR dsRNAs compared to control transfected cells (**Figure 5-4A**). In order to determine if knockdown of both apoER2 and VLDLR proteins alter aPL-mediated eNOS antagonism, the effect of aPL on eNOS activity was tested in cells transfected with either control siRNA or apoER2/VLDLR siRNAs. BAEC were transfected with dsRNAs and 24h post-transfection, the cells were pretreated with NHIgG (100 μg/ml) or aPL (100 μg/ml) for 15 min and incubation with VEGF was for an additional 15 min. In the presence of control siRNA, aPL inhibited eNOS activation (**Figure 5-4B**). Similarly, after knockdown of both apoER2 and VLDLR by siRNA, aPL antagonized eNOS activation by VEGF (**Figure 5-4B**). Thus, loss of both apoER2 and VLDLR by siRNA has no effect on aPL-mediated eNOS antagonism.

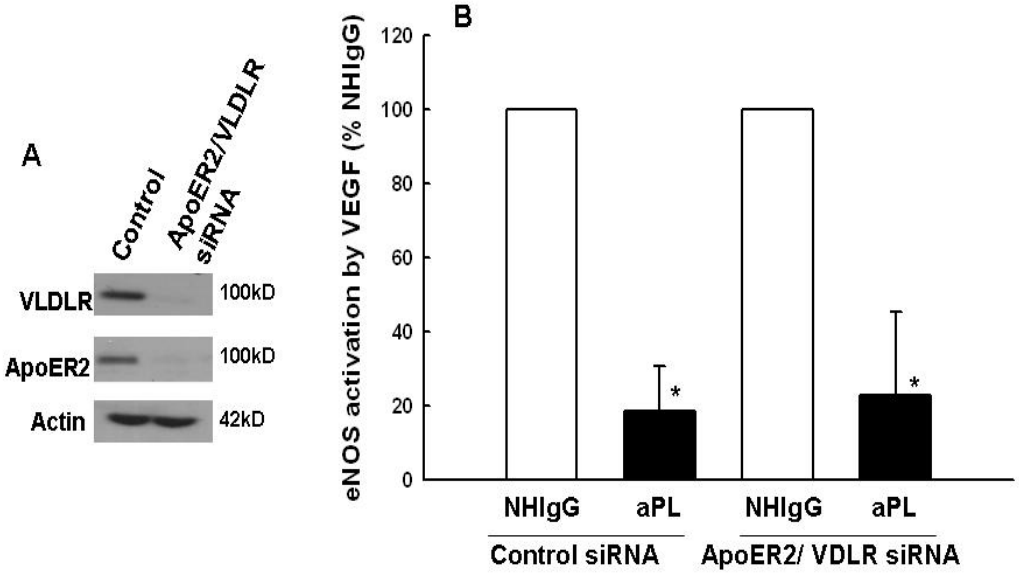

Figure 5-4: Loss of both apoER2 and VLDLR by siRNA does not alter aPL-mediated eNOS antagonism.
(**A**) BAEC were transfected with scrambled (control) or apoER2 and VLDLR siRNA and 24 h later apoER2 and VLDLR protein expression was assessed by immunoblotting. (**B**) BAEC were transfected with scrambled (control) or apoER2 and VLDLR siRNA and 24 h later eNOS activation in response to VEGF (100ng/ml, 15 mins) was assessed following preincubation for 15 min with NHIgG (100μg/ml) or aPL(100μg/ml).

Since residual receptors may be present following attempted siRNA knockdown, a genetic strategy was then taken and the impact of aPL on Ach-mediated changes in carotid artery vascular conductance was measured in apoER2$^{+/+}$ vs. apoER2$^{-/-}$ mice. The results described below are for four mice in

each group. At baseline, acetylcholine caused a dose-dependent increase in vascular conductance in both types of mice (**Figure 5-5, ●**). Administration of aPL (2mg) blunted the Ach response (**Figure 5-5A,** ○) in apoER2$^{+/+}$ mice. In contrast, aPL administration had no effect on the Ach response (**Figure 5-5B,** ○) in the apoER2$^{-/-}$ mice. Ach-mediated increases in vascular conductance in both groups were blunted by administration of the eNOS antagonist L-NAME (Figure 5-5, ▼). Thus, aPL-mediated eNOS antagonism requires apoER2.

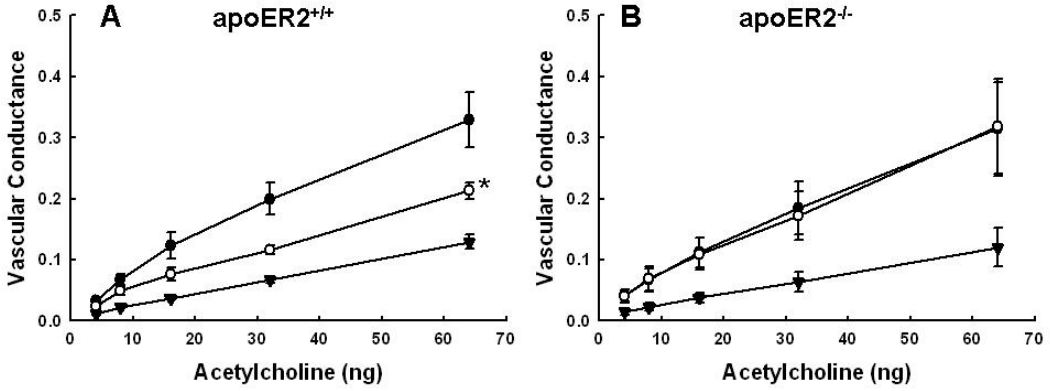

Figure 5-5: aPL-mediated eNOS antagonism is mediated by apoER2.
Male apoER2$^{+/+}$ (**A**) and male apoER2$^{-/-}$ (**B**) mice were instrumented and changes in carotid vascular conductance in response to eNOS activation were compared before and after administration of aPL (2mg, IV). Dose responses to Ach were determined sequentially at baseline (control, ●), 60 minutes after administration of aPL (○) and 10 minutes after L-NAME administration (▼). Values are mean $\pm$ SEM, n=4/ group. * $p<0.05$ vs control (two-way ANOVA).

Conclusions

β2GPI/ antibody complexes can activate platelets. A splice variant of apolipoprotein E receptor 2, apoER2' has been demonstrated to be involved in the binding of antibody complexed β2GPI to human platelets (Lutters et al., 2003). Dimeric β2GPI can also bind to other members of the LDLR family including very low density lipoprotein receptor (VLDLR), LDLR related protein (LRP), and megalin (Pennings et al., 2006). apoER2 and VLDLR are expressed in the endothelium. Since aPL antagonism of eNOS requires β2GPI, we tested the requirement for one/ more receptors of the LDLR family for this antagonism.

Using the inhibitor of the LDL receptor family RAP we have shown that the effects of aPL on eNOS activity are antagonized by the presence of RAP but not in the presence of GST controls. This indicates that one/ more members of the LDLR family are involved in aPL-mediated eNOS antagonism. To determine the member/s of the LDL receptor family involved in aPL action, we tested the involvement of apoER2 and VLDLR using siRNA. We obtained effective knockdown of either apoER2 or VLDLR in BAEC, and aPL inhibition of eNOS activity was unaffected. Both apoER2 and VLDLR can act as receptors for the glycoprotein reelin and knockout of any one of the receptors does not affect reelin function, indicating that apoER2 and VLDLR can compensate for each other in reelin signaling (May et al., 2005). To recapitulate the phenotype of the reelin knockout mice (reeler mice) (D'Arcangelo et al., 1995) both apoER2 and VLDLR

have to be absent (Trommsdorff et al., 1999). We therefore also tested the effect of aPL on eNOS activation after knockdown of both receptors. In endothelial cells, knockdown of apoER2 and VLDLR by siRNA did not rescue aPL-mediated eNOS antagonism. It is possible that siRNA did not sufficiently knockdown the target protein/s and residual receptor was sufficient to transduce the signal to inhibit eNOS activation. To overcome this problem, we examined the effect of aPL on Ach-induced changes in carotid vascular conductance in apoER2$^{-/-}$ mice vs. apoER2$^{+/+}$ mice. Whereas aPL action was quite apparent in apoER2$^{+/+}$ mice, aPL did not attenuate Ach-mediated increases in carotid vascular conductance in apoER2$^{-/-}$ mice. These observations indicate that apoER2 is required for aPL-mediated eNOS antagonism. Further studies using vascular conductance need to be carried out to test the involvement of other receptors in the LDLR family known to interact with β2GPI, namely VLDLR, LRP and megalin. The effect of RAP on carotid vascular conductance can also be tested *in vivo* in mice. We would expect RAP to reverse aPL attenuation of Ach-mediated increases in vascular conductance.

Now that atleast one important receptor for aPL action in endothelium has been identified, further studies need to be carried out to determine the signaling pathway between apoER2 and the attenuation of eNOS S1179 phosphorylation that underlies eNOS antagonism and its downstream consequences.

Chapter 6

General Conclusions and Future Directions

The antiphospholipid syndrome is an autoantibody-mediated disease characterized by recurrent fetal loss, vascular thrombosis and thrombocytopenia occurring in the presence of circulating autoantibodies (aPL) (Miyakis et al., 2006). Patients with APS also have an increased risk of coronary artery disease and myocardial infarction. The presence of aPL has been shown to be directly responsible for the manifestations of the disease. However, the pathogenic mechanisms of the disease are not well understood. The endothelium is a critical direct target of aPL and endothelial cell dysfunction can be caused by interaction of aPL with the surface phospholipids leading to enhanced monocyte adhesion (Del Papa et al., 1995; Pierangeli et al., 1999; Riboldi et al., 2003; Simantov et al., 1995). aPL also causes increased production of proinflammatory and prothrombotic factors by the endothelium (Amengual et al., 1998; Branch and Rodgers, 1993; Conti et al., 2003; Del Papa et al., 1997; Del Papa et al., 1995). Initially thought to recognize anionic phospholipids, it is now apparent that the major antigenic target of aPL on the endothelium is cell surface proteins, predominantly β2GPI (Galli et al., 1990; McNeil et al., 1990).

NO is a key determinant of vascular health regulating several physiological processes including thrombosis, interaction of endothelium and monocytes, endothelial cell migration and proliferation, vascular permeability, and smooth muscle cell growth and migration. Impaired NO bioavailability represents a central feature of endothelial dysfunction in vascular thrombosis and is important for development of atherogenesis. eNOS is the primary source of bioavailable NO in the vasculature. The enzymatic activity of eNOS is modulated by phosphorylation of the enzyme and various protein-protein interactions (Fleming and Busse, 1999; Fulton et al., 2001; Kone, 2000; Shaul, 2002; Venema, 2002).

In the present study, we have demonstrated that aPL inhibit the activation of eNOS by different agonists, and that this is due to impaired eNOS S1179 phosphorylation. To link the effect of aPL on eNOS activation to a change in endothelial cell function, we have shown that aPL-induced declines in NO production underlie the promotion of monocyte adhesion by aPL *in vitro*. We have also demonstrated in a mouse model that aPL antagonizes eNOS activation *in vivo*.

Complement is required for the induction of fetal loss *in vivo* by aPL antibodies (Holers et al., 2002). However, we have demonstrated that aPL-mediated eNOS inhibition does not require complement. Studies have also shown that aPL exerts its thrombogenic effects via the crosslinking of Fcγ receptors and

polymorphisms of FcγRIIA can be used as a clinical predictor for increased thrombosis risk (Arvieux et al., 1995; Karassa et al., 2003; Sammaritano et al., 1997). Other studies have been carried out which contradict these results (Atsumi et al., 1998; Girardi et al., 2003). Here we have demonstrated that aPL-mediated eNOS inhibition is via the F(ab')2 fragment of aPL and does not require the Fc fragment of the antibody, thus eliminating the possibility of the requirement for Fc receptors.

We have also demonstrated a requirement for β2GPI for aPL action on eNOS by two different approaches. We have shown that upon serum starvation aPL antagonism of eNOS is abolished and that a β2GPI monoclonal antibody has actions similar to aPL. It has been determined using patient samples and β2GPI domain deletion mutants that aPL antibodies recognize an epitope in domain I of β2GPI. Thromboembolic complications are best associated with patient antibodies directed against domain I of β2GPI (de Groot and Derksen, 2005). Antibodies directed against domain I of β2GPI also show a positive β2GPI-dependent lupus anticoagulant (LAC) assay indicating that these antibodies are pathogenic (de Groot and Derksen, 2005). We have shown that domain I of β2GPI, that is recognized by pathogenic aPL, is important for eNOS inhibition, thereby indicating the potential pathological significance of eNOS inhibition.

We also tested the requirement for members of the LDLR family. It has been shown that dimeric, but not monomeric, β2GPI binds to different members of the LDL receptor family including apoER2, VLDLR, LRP and megalin (Pennings et al., 2006). Our results using the universal inhibitor of the LDL receptor family RAP show that one or more of the LDL receptor family members are involved in aPL-mediated eNOS inhibition. Studies of carotid vascular conductance in wild type and apoER2 knockout mice further indicate that apoER2 is required for aPL-mediated eNOS antagonism. The signaling cascade mediating aPL inhibition of eNOS activation leading to endothelial dysfunction is outlined in **Figure 6-1**.

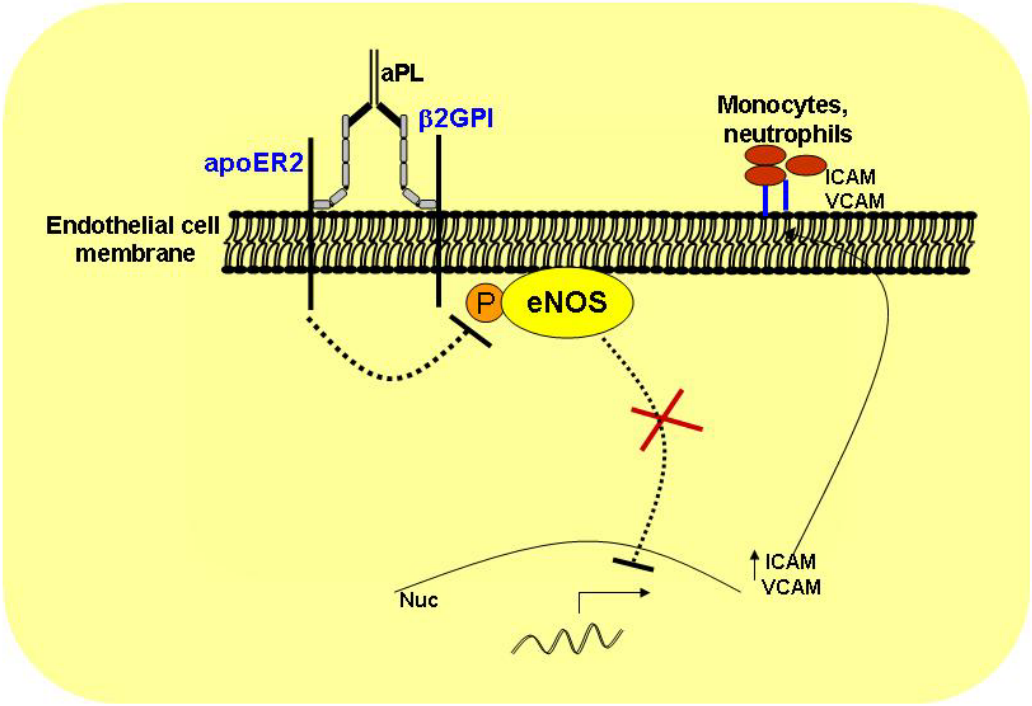

Figure 6-1: aPL inhibits eNOS activation via β2GPI and apoER2.
Binding of aPL via its F(ab')2 region to domain I of β2GPI causes dimerization of β2GPI and through apoER2 inhibits eNOS activation via changes in eNOS S1179 phosphorylation (dotted line). The attenuation of eNOS activity decreases NO production, leading to increased synthesis and expression of adhesion molecules on the surface of endothelial cells. Increased expression of adhesion molecules causes an endothelial proadhesive phenotype leading to endothelial dysfunction.

Further studies need to be carried out to delineate the remaining components of the pathway of aPL-mediated eNOS inhibition. The mechanism by which the aPL/β2GPI/apoER2 complex decreases in eNOS S1179 phosphorylation need to be further investigated. One mechanism for the decrease

in eNOS S1179 phosphorylation could be an increase in the activity of the phosphatase PP2A that causes eNOS dephosphorylation. CRP-mediated decreases in eNOS S1179 phosphorylation and consequently eNOS activity are via modulating PP2A activation (Mineo et al., 2005). However, the phosphorylation and activation of Akt, which is also dephosphorylated by PP2A, is unaltered in the presence of aPL. These observations mimic those with endostatin, which attenuates eNOS activity and blocks VEGF-induced NO synthesis and endothelial cell migration via blunting eNOS S1179 phosphorylation. Endostatin decreases eNOS S1179 phosphorylation by increasing the activity of PP2A. Similar to aPL, endostatin has no effect on Akt phosphorylation (Urbich et al., 2002).

Another mechanism by which aPL may inhibit eNOS S1179 phosphorylation is by altered localization of the enzyme thus rendering it unavailable for phosphorylation by Akt. Since the plasma membrane is the most effective site of eNOS phosphorylation and activation, altered enzyme localization may be a mechanism of aPL-mediated attenuation of eNOS activity (Gonzalez et al., 2002; Shaul, 2002; Zhang et al., 2006).

The potential role of other candidate receptors in the LDL receptor family, namely VLDLR, LRP1 or megalin, need to be determined. Studies also have to be carried out to determine the signaling mechanism that enables apoER2 and possibly other LDLR family members to inhibit eNOS activity. Dimerization or

clustering of apoER2 in neurons causes activation of the Src family of kinases leading to tyrosine phosphorylation of disabled 1 (Dab1) and activation of downstream signaling and PI3K/ Akt activation (Strasser et al., 2004). How apoER2 potentially modifies signaling events in endothelium is entirely unknown. Since both LRP1 and megalin have functions in both signaling and endocytosis (May et al., 2005), it would be interesting to speculate if interaction of aPL with one of these receptors causes internalization of eNOS through endocytosis, thus rendering the enzyme incapable of being phosphorylated. The role of other proteins that have been shown to be involved in aPL-mediated endothelial dysfunction, including annexin A2, also need to be determined in aPL-β2GPI mediated eNOS inactivation. Other candidate receptors that may be involved in aPL action include members of the toll like receptor (TLR) family (Meroni et al., 2004), though our results with the inhibitor of LDLR family (RAP) and genetic apoER2 deletion indicate otherwise. Once the different components of the signaling cascade between aPL, apoER2 and possible other receptors and eNOS have been identified, reconstitution in a cell culture system could confirm the requirement of the individual proteins in aPL-mediated eNOS attenuation.

To further confirm the physiological relevance of aPL inhibition of eNOS, studies need to be carried out to determine the effect of aPL on eNOS-mediated endothelial cell migration and reendothelialization in mice. CRP inhibition of eNOS leads to decreased endothelial cell migration and decreased

reendothelialization in mice (Schwartz et al., 2007), and it is possible that aPL also contributes to vascular disease by blunting endothelial cell migration.

Studies in the lab of our collaborator, Dr. Craig Morrell at Johns Hopkins University, MD have recently demonstrated that aPL inhibits eNOS activation in platelets *in vitro* and *in vivo* in a thrombosis model. Their studies with RAP in platelets demonstrate the requirement for a member of the LDLR family for aPL-mediated eNOS antagonism similar to our studies in the endothelium. Collectively our results demonstrate that aPL inhibits eNOS activation both in the endothelium and in platelets. This decrease in NO production by both the endothelium and platelets potentially leads to increased endothelium-leukocyte interaction, platelet-leukocyte interaction and platelet aggregation. Increased thrombus formation, due to lack of bioavailable NO, may cause increased cardiovascular risk in APS patients.

The antiphospholipid syndrome is characterized by intrauterine growth retardation (IUGR) and pregnancy loss. Human umbilical vein endothelial cells (HUVECs) isolated from IUGR fetuses exhibit reduced L-arginine transport and reduced NO production (Casanello and Sobrevia, 2002). Loss of NO by inhibiting eNOS activity may represent a mechanism by which aPL causes IUGR. It would be therapeutically useful to determine if intervention with a NO donor reversed the manifestations of aPL in pregnancy. This can be carried out by testing the effect of a NO donor in the murine model of APS developed in the laboratory of

Dr. Jane Salmon at the Hospital for Special Surgery and the Weill Medical College of Cornell University. One of the potential caveats of this intervention could be the increased vasodilation and decreased blood pressure due to NO administration.

In conclusion, our studies are the first to link aPL-mediated increases in monocyte adhesion to decreases in bioavailable NO and to decrease in eNOS activity. Our work identifying a requirement for endothelium-bound β2GPI and for apoER2 further elucidates the most proximal mechanism by which aPL inhibits the normal functioning of the endothelium. We propose that these novel processes contribute to the greater cardiovascular disease risk and adverse pregnancy outcomes in patients with APS.

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Vitae

Sangeetha Ramesh was born in Mumbai, India on June 29, 1977, the daughter of Vasanthi Ramesh and Ramesh B Iyengar. She received a degree in Bachelor of Science with a double major in Microbiology and Biochemistry from the University of Mumbai, India in May 1997. She entered the Institute of Science, Mumbai and received the degree of Masters of Science in Biochemistry from the University of Mumbai, India in May 1999. In August 2000, she entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. She joined the lab of Dr. George DeMartino in the Department of Physiology and received the degree of Masters of Science in Integrative Biology in May 2004. She joined the lab of Dr. Philip Shaul in the Department of Pediatrics in May 2004. She married Karthik Rajagopal in June, 2001 and has 2 daughters Anika and Isha.

Permanent Address: 2720 Meadow Wood Dr
Flower Mound, TX 75022