Fluvastatin increases the expression of adhesion molecules, monocyte chemoattractant protein-1 and tissue factor in HUVEC stimulated by patient IgG fractions containing antiphospholipid antibodies

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Abstract

The presence of antiphospholipid antibodies (APLA) is associated with an increased risk of recurrent thrombosis and pregnancy loss. APLA are able to activate endothelial cells (EC) and induce an increase in the expression of inflammatory marker proteins, such as leukocyte adhesion molecules, tissue factor or the monocyte chemoattractant protein-1 (MCP-1). Our objective was to investigate the effect of statins on EC activation induced by APLA in vitro. IgG was purified from the plasma of six patients with APLA and from healthy controls. EC were incubated with patient IgG or with control IgG, in the presence or absence of 5μM of fluvastatin, and expression of the leukocyte adhesion molecules, VCAM-1 and E-selectin, analyzed by flow cytometry and by quantitative reverse transcriptase-PCR (QRT-PCR). The expression of tissue factor and the chemokine MCP-1 was analyzed by QRT-PCR alone. Incubation of EC with patient IgG increased the expression of VCAM-1, E-selectin, tissue factor and MCP-1. Prior treatment of the cells with fluvastatin further increased the expression of these proteins. The fluvastatin effect was reversed [...]
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Summary
The presence of antiphospholipid antibodies (APLA) is associated with an increased risk of recurrent thrombosis and pregnancy loss. APLA are able to activate endothelial cells (EC) and induce an increase in the expression of inflammatory marker proteins, such as leukocyte adhesion molecules, tissue factor or the monocyte chemoattractant protein-1 (MCP-1). Our objective was to investigate the effect of statins on EC activation induced by APLA in vitro. IgG was purified from the plasma of six patients with APLA and from healthy controls. EC were incubated with patient IgG or with control IgG, in the presence or absence of 5μM of fluvastatin, and expression of the leukocyte adhesion molecules, VCAM-1 and E-selectin, analyzed by flow cytometry and by quantitative reverse transcriptase-PCR (QRT-PCR). The expression of tissue factor and the chemokine MCP-1 was analyzed by QRT-PCR alone. Incubation of EC with patient IgG increased the expression of VCAM-1, E-selectin, tissue factor and MCP-1. Prior treatment of the cells with fluvastatin further increased the expression of these proteins. The fluvastatin effect was reversed by co-incubation with mevalonate or geranylgeranylpyrophosphate and mimicked by the geranylgeranyl transferase inhibitor GGTI-286. Our results show that in cultured human EC, statins increase the extent of inflammatory activation induced by APLA. This effect appears to be mediated by an inhibitory effect of statins on one or more geranylgeranylated protein(s).

Keywords
Antiphospholipid antibodies, endothelial cells, statins, tissue factor, cell adhesion molecules

Introduction
The vascular endothelium represents a dynamic barrier between blood and surrounding tissue and is a major regulator of haemostasis, leukocyte migration and vascular tone. In response to activation by inflammatory cytokines, endothelial cells (EC) increase the expression of proteins involved in leukocyte recruitment, adhesion and transmigration leading to a proinflammatory phenotype. Among these proteins, adhesion molecules such as VCAM-1, ICAM-1 and E-selectin, or chemokines, such as monocyte chemoattractant protein-1 (MCP-1) play an important role (1, 2). In addition, expression of tissue factor is increased in activated EC (3). In patients, the presence of antiphospholipid antibodies (APLA) is associated with an increased risk of thrombosis and recurrent fetal loss. The occurrence of APLA with clinical manifestations characterizes the antiphospholipid syndrome (APS). Several studies have shown that incubation of EC with APLA leads to an increased expression of leukocyte adhesion molecules and of tissue factor, and enhance monocyte adhesion in vitro (4–6). Furthermore, in vivo, APLA increase the adhesion of leukocytes to the endothelium in the cremaster muscle microcirculation model in mice (7, 8). In patients with APS, plasma levels of circulating markers of EC activation are increased (9, 10). These observations suggest that in vivo and in vitro APLA induce EC activation, which may contribute to their pathological effects.

Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) are cholesterol-lowering drugs and are widely used in the treatment of hyperlipidemia to prevent cardiovascular com-
plications. They inhibit the formation of mevalonate, a rate limiting intermediate in the biosynthesis not only of cholesterol but also of farnesyl- and geranylgeranyl-pyrophosphate, which provide lipid anchors that are required for the biological activity of small GTP binding proteins. More than one hundred small GTP binding proteins have been identified. They are classified into five families, including the Ras and Rho protein families, which play key roles in the regulation of gene expression and of the organization of the cytoskeleton. The inhibition of the farnesyl- or geranylation of small GTP binding proteins may underlie many of the pleiotropic effects of statins (11). The anti-inflammatory effect of statins has been reported in several studies, and may contribute to their beneficial effects. However, the anti-inflammatory effects of statins on cytokine-mediated induction of leukocyte adhesion molecule expression in EC remain controversial. Several groups reported that statins inhibit adhesion molecule expression induced by proinflammatory cytokines in vitro (5, 12). In contrast, others have shown that statins increased adhesion molecule expression induced by TNFα (13–16). Until now, few studies focused on the possible effect of statins on EC activation induced by APLA. In vitro, it was shown that fluvastatin, and to a lesser extent simvastatin, reduced both the expression of U937 monocyte-like cells to APLA-treated human umbilical vein endothelial cells (HUVEC) and the expression of E-selectin and ICAM-1 (5). Furthermore, in an in vivo study, fluvastatin treatment reduced thrombus size, the number of adherent leukocytes and soluble ICAM-1 concentrations in mice treated with APLA (17).

The aim of our study was to investigate the influence of statins on the synthesis and expression of several inflammation-related proteins by APLA-activated EC. We show that, in EC treated with IgG from patients with APLA, statins further increased the expression of the leukocyte adhesion molecules VCAM-1 and E-selectin, of the monocyte chemoattractant protein MCP-1 and of tissue factor. The statin-mediated increase was prevented by the addition of mevalonate or GGPP and mimicked by the addition of the geranylgeranyl transferase inhibitor GGTI-286.

**Material and methods**

**Reagents**

Fluvastatin and the geranylgeranyl transferase inhibitor-286 (GGTI-286) were obtained from Calbiochem (San Diego, CA, USA). Mevalonic acid lactone and geranylgeranyl-pyrophosphate (GGPP) were obtained from Sigma (St. Louis, MO, USA). Mevalonic acid was activated by treatment with 0.1 M NaOH at 50°C for 2 hours; then the pH was adjusted to 7.4 with HCl. Monoclonal anti-VCAM-1 (anti-CD106), and anti-E-selectin (anti-CD62E/CD62P) antibodies were purchased from Serotec (Oxford, UK). FITC-conjugated goat anti-mouse antibodies used as secondary antibodies for flow cytometry analysis, was from Cappel Organon Technika (Durham NC, USA).

**Patients, blood samples and isolation of IgG**

Six patients with APLA, and suffering from thrombotic complications and/or pregnancy morbidity, were chosen among a group of patients evaluated in a previous study for the ability of their IgG to induce cell activation in vitro (18). The clinical and serological characteristics of these patients are summarized in table 1. IgGs were isolated from the patient plasmas on Protein A CL-4B Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Protein levels were measured by the bichinchoninic acid protein assay (BCA Protein assay, Pierce, Rockford IL, USA). Endotoxin levels, measured by the Limulus Amebocyte Lysate Endochrome Assay (Charles River Laboratories, Charleston SC, USA), were found to be below the detection limit (0.25 EU/ml) for all IgG samples at the concentration used in the activation assays. The negative control antibody consisted of IgG prepared from a pool of ten healthy donors.

**APLA assays**

The titers of anticardiolipin IgG in the patient plasmas were determined as previously described and higher than 5 GPL Units (19). Levels of anti-β2GP1 IgG in plasma were evaluated by ELISA (20) and were positive in all patients. Lupus anticoagulant was evaluated according to the International Society of Thrombosis and Haemostasis criteria using aPTT-LA and Staclot LA (Stago, Anières, France) and dilute Russell’s viper venom tests (Dade Behring, Marburg, Germany). Lupus anticoagulant was present in four out of 6 patients. All APLA assays were repeated at least once at an interval of at least six weeks. The purified IgG fractions were analyzed for anticardiolipin and anti-β2GP1 activity and found to be positive.

**Isolation and culture of endothelial cells**

Endothelial cells (HUVEC) were isolated from umbilical cord veins and cultured as described previously (21, 22). Cells were passaged by 0.05% trypsin-0.02%EDTA (Seromed Biochrom KG, Berlin, Germany) treatment at a split ratio of 1:2, and used between passages 1 to 4. The cell culture medium was RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA, USA), 90 µg/ml heparin (Roche, Reinach,

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age</th>
<th>Clinical manifestations</th>
<th>IgG ACL</th>
<th>IgG antiβ2GP1</th>
<th>LA</th>
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<td>P1</td>
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<td>Thromboembolism, thrombocytopenia</td>
<td>&gt;60</td>
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</tr>
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<td>P6</td>
<td>F/30</td>
<td>Fetal loss, skin lesion</td>
<td>36</td>
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</tbody>
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*ACL IgG are expressed as GPL Units (normal values<5GPL) ; SLE for systemic lupus erythematosus, LA for lupus anticoagulant.
Switzerland), 15 µg/ml EC growth supplement (Upstate Biotechnology, Lake Placid NY, USA), 10 mM HEPES, 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco BRL-Life Technologies, Rockville MD, USA). The FCS was depleted by treatment for one hour at 56°C.

Stimulation of endothelial cells with APLA and treatment with fluvastatin and inhibitors

HUVEC were grown in 24-well plates. After reaching confluency, the cells were incubated in complete medium overnight with 5 µM fluvastatin with or without mevalonate (400 µM) or GGPP (15 µM). Then, culture medium was replaced by RPMI 1640, 20% FCS, 10mM Hepes, 100 IU/ml penicillin and 100 µg/ml streptomycin, containing, where appropriate, fluvastatin, mevalonate and GGPP. Cells were stimulated with purified IgG-APLA (0.5 mg/ml). For negative controls cells were incubated in the same conditions with medium alone or normal human serum IgG (0.5 mg/ml). Twenty-four hours after stimulation with IgG-APLA or control IgG, cells were isolated for flow cytometry analysis or mRNA extraction.

Similar experiments were performed with HUVEC after prior incubation for 48 hours with GGTI-286 (10 µM). Fresh GGTI-286 solutions were added after 24 hours. Thereafter, cells were treated with IgG-APLA or control IgG for 24 hours and cells or mRNA isolated for further analysis.

Analysis of adhesion molecule expression on EC by flow cytometry

Cells were examined for surface antigen expression by flow cytometry as previously described (15). The cells were washed in RPMI and incubated for 1 hour at 4°C with mouse monoclonal antibody (anti-E-selectin or anti-VCAM-1) at 10 µg/ml in PBS (Gibco, BRL-Life Technologies, U.K.), 5% FCS and 0.02% sodium azide. Cells were fixed in 2.5% formaldehyde, 2% glucose, 0.02% sodium azide in PBS. Propidium iodide (10 µl of 50 µg/ml) was added to each sample. Non-viable propidium iodide-positive cells were gated out. Cell fluorescence was measured on a Becton Dickinson FACScan flow cytometer (San Jose CA, USA). A total of 10000 viable cells were analysed per experimental sample. Data were analysed using Cellquest software (Becton Dickinson).

RNA extraction and reverse transcription

Total RNA was extracted from cells, and treated with DNaseI, using RNeasy® kits (Qiagen GmbH, Hilden, Germany). The RNA yield and purity were assessed by absorbance measurements at 260 and 280 nm. From each sample, 1 µg of RNA was used as template for cDNA synthesis using the ImPromII™ Reverse transcription system (Promega, Madison WI, USA).

Quantitative real time reverse transcriptase PCR assay

Relative mRNA levels of VCAM-1, E-selectin, MCP-1, and tissue factor were quantified by real-time PCR using the cDNA templates, with an Applied Biosystems Prism 7000 instrument and SYBR-green® PCR master mix reagent (Applied Biosystems, Warrington, UK). Specific forward and reverse primers used were respectively:

- VCAM-1: CATGGAATTCGAACCAAACA and GACCAA-GACGGTTGTATCTCTGG;
- E-selectin: ATCATCCTGCAACTTCACCa nd ACACCTCAC-GGTTGTATCTCTGG;
- MCP-1: GTGACCTGCTGCTGCTATTAC and ACCACCTCAC-AAAACCCTC;
- tissue factor: CTACTGTTTCAGTGTTCAAGCAGTGA and CAGTGCAATATAGCATTTGCAGTAGC. Glyceraldehyde-3-phosphate dehydrogenase, used as the control gene, was amplified and quantified using as a forward primer GGTGAAAGTCTGGAGTCAAC and reverse primer CCAATGGAATCATATTG.

Data were analyzed using the ∆C_T method and Applied Biosystems Prism software, according to the manufacturers instructions and expressed as fold increase over control cells to which no IgG or other agents had been added.

Statistical analysis

The effect of fluvastatin on the cell surface expression of VCAM-1 and E-selectin and on mRNA synthesis of VCAM-1, E-selectin, tissue factor and MCP-1 was assessed by the paired student’s t-test. The statistical analysis of the effect of mevalonate was also performed by the paired student’s t-test.

Results

Effect of fluvastatin on adhesion molecules and tissue factor mRNA expression in non stimulated HUVEC

Fluvastatin alone had no effect on VCAM-1 or E selectin expression as measured by flow cytometry analysis. We made a
time course on the effect of 5 μM fluvastatin on mRNA levels of inflammatory marker proteins. The maximum effect was observed at 16 h: indeed 5 μM fluvastatin induces a 1.6 to 3.6-fold increase in VCAM-1, E selectin, MCP-1 and tissue factor mRNAs (Fig. 1). The increase was reversed by cotreatment with mevalonate (not shown).

**Fluvastatin increases the surface expression of VCAM-1 and E-selectin on HUVEC stimulated by IgG from patients with APLA**

IgG was isolated from six patients positive for APLA (IgG-APLA). In preliminary experiments these IgGs were shown to induce a significant increase in VCAM-1 expression as compared with control IgG. Treatment of HUVEC for 16 h with 5 μM fluvastatin prior to IgG-APLA stimulation for 24 h resulted in increased expression of VCAM-1 or E-selectin. Figure 2 shows a representative flow cytometry profile of VCAM-1 (A) and E-selectin (B) expression obtained with cells incubated with 500 μg/ml of IgG-APLA from patient 1 in the presence or absence of fluvastatin. For all six IgG-APLA samples, we observed an increase in VCAM-1 and in E-selectin expression in fluvastatin-treated cells, compared with cells treated with IgG-APLA alone (p < 0.01 Fig 2C and p < 0.01 Fig 2D, respectively, paired student’s t-test). Fluvastatin was used at 5 μM, a concentration that is not cytotoxic (5).

**Effect of fluvastatin on the expression of VCAM-1, E-selectin, MCP-1 and tissue factor mRNA by IgG-APLA-activated HUVEC**

We further investigated the effect of fluvastatin on IgG-APLA-treated HUVEC by measuring the mRNA levels of various inflammation-related proteins. HUVEC were incubated overnight with or without fluvastatin and then stimulated with IgG-APLA for 24 h (from 4 out of the 6 IgG-APLA samples). Total RNA samples were isolated, reverse transcribed and quantitative RT-PCR analysis performed for VCAM-1, E-selectin, MCP-1 and tissue factor. Levels of each mRNA were similar in non-treated HUVEC and in HUVEC treated with control IgG. As shown in Figure 3, mRNA levels of VCAM-1, E-selectin, tissue factor and MCP-1 increased after incubation with IgG-APLA from pa-
Patients, compared to untreated HUVEC. Simultaneous stimulation of HUVEC with fluvastatin and IgG-APLA further increased mRNA expression of the four molecules (respectively p<0.05 for VCAM-1, p<0.01 for E-selectin, p<0.005 for MCP-1 and p<0.025 for tissue factor, paired student’s t-test, n=4).

The effect of fluvastatin on IgG-APLA-induced overexpression of adhesion molecules, tissue factor and MCP-1 are related to the isoprenoid pathway

The addition of 400 μM mevalonate partially reversed the over-expression of VCAM-1 and E-selectin observed by flow cytometry in fluvastatin- and IgG-APLA -treated HUVEC (Fig. 2). This confirms that the effect of fluvastatin is due to the inhibition of HMG-CoA reductase. The difference between mean fluorescence values obtained with the combination IgG-APLA and fluvastatin with or without mevalonate assessed by a paired student’s t-test had a p value <0.005 for VCAM-1 and p<0.001 for E-selectin (n=6).

In a previous study, we have shown that the increasing effect of statins (simvastatin and fluvastatin) on adhesion molecule expression induced by TNF-α was completely reversed by GGPP (15 μM) whereas FPP had only a partial effect, even when higher doses were used (15). Based on this result, we studied the effect of GGPP on the fluvastatin-mediated increase in VCAM-1, E-selectin, MCP-1 and tissue factor expression induced by IgG-APLA. Fig 2A and B shows the results obtained by flow cytometry analysis with IgG-APLA from one of the patients. (Patient 1). These results show that 15 μM GGPP reverses the effect of fluvastatin to a similar extent as mevalonate. Treatment of HUVEC with GGTP-286 induced a significant increase in E-selectin or VCAM-1 expression induced by IgG-APLA. Similar results were obtained for the six IgG-APLA samples (Fig 2C and D).

We studied the effect of mevalonate, the isoprenoid intermediate GGPP as well as GGTI-286 on the expression of VCAM-1, E-selectin, MCP-1 and tissue factor mRNA after stimulation of EC with IgG-APLA (Fig. 3). The enhancing effect of fluvastatin on the mRNA levels was reverted by coinubation with 400 μM mevalonate or 15 μM GGPP. Similar profiles were obtained for the four IgG-APLA-positive patient IgG samples tested. Coincubation with GGTI-286 had a similar effect as fluvastatin on the IgG-APLA induced mRNA overexpression of the four molecules tested.

Discussion

In this study we show that APLA from patients increase the expression of leukocyte adhesion molecules, of MCP-1 and of tissue factor and that fluvastatin further enhances this stimulatory
effect. The effects observed for APLA on protein expression in EC confirm the results of previous studies (4–6, 23). Induction of an adhesive phenotype on endothelial cell is not attributable to contaminating endotoxins since purified IgG from healthy donors do not induce adhesion molecule expression and Limulus assays on sample preparations were confirmed to be endotoxin negative. We therefore conclude that the effect of APLA on EC is antibody specific.

As the so-called pleiotropic „anti-inflammatory“ activity of statins represent an attractive mechanism for their beneficial clinical effects in addition to their well known cholesterol lowering effects (11, 24–26), we examined whether fluvastatin modified the APLA-induced up-regulation of VCAM-1, E-selectin, tissue factor and MCP-1. We observed that preincubation with fluvastatin led to an additional increase in each mRNA in response to IgG-APLA treatment of HUVEC. The effects of fluvastatin on the expression of VCAM-1 and E-selectin, induced by IgG-APLA, were comparable with our previous observations on the effect of simvastatin and fluvastatin on the expression of adhesion molecules induced by TNFα or LPS (15).

Several studies have been conducted to evaluate the effect of statins on EC activation induced by inflammatory cytokines with conflicting results. Part of these studies reported that statins were able to decrease adhesion molecule expression and macrophage adhesion on EC stimulated by TNFα or LPS (5,12). Furthermore, statins were reported to decrease the expression of tissue factor on macrophages stimulated with LPS (27). From this, an attractive mechanism for a protective effect of statins has been proposed, in which statins decrease the inflammatory response of the endothelium resulting in a lower leukocyte adhesion and a lower tissue factor expression both on the endothelium and the attached leukocytes. These effects would then lead to a lower procoagulant activity in inflammatory regions. In support for this hypothesis, fluvastatin was reported to reduce tissue factor expression and macrophage accumulation in carotid lesions of cholesterol-fed rabbit (28). In contrast, several other studies rather observed that statins or geranylgeranyltransferase inhibitors rather potentiated the cytokine-mediated increase in adhesion molecules (13–16). Several reasons for these discrepancies were put forward without finding a definitive explanation: the use of different compounds of the statin family, the diverse agonists employed in activating the cells or differences in the techniques of evaluating adhesion molecules expression (29). One recent study reported results that may reconcile, at least in part, the discrepancies (16). In this study statins did increase adhesion molecule expression on TNF-treated EC, but decreased adhesion molecule clustering and monocyte-like cell adhesion. It is known that adhesion molecule clustering and leukocyte adhesion are dependent on members of the Rho family of GTP-binding proteins (30), which require geranylgeranylation for expression of their activities. In this model the effect of statins or geranylgeranyltransferase inhibitors on leukocyte adhesion is due to their inhibition of these small GTP-binding proteins, rather than to an inhibition of adhesion molecule responses to inflammatory mediators.

The effect of statins on the inflammatory properties of APLA was investigated in two previous studies: in an in vitro experiment, Meroni (5) reported that simvastatin and fluvastatin decreased the EC expression of E-selectin and ICAM-1 in response to immunopurified APLA. In a microcirculation model of exposed cremaster muscle, mice receiving intraperitoneally APLA and treated with fluvastatin showed smaller thrombi, a reduced number of adherent leukocytes and decreased levels of circulating soluble ICAM-1 compared with animals receiving APLA without fluvastatin (17). These two results implied a beneficial effect of statins on the in vivo and in vitro response to APLA. Our findings contradict these results. We observed that fluvastatin alone induced a small increase in VCAM-1, E-selectin, MCP-1 and tissue factor mRNA. However, this effect was not detected at the cell surface by flow cytometry. Kiener et al (31) found that treatment of human monocytes with lipophilic statins alone stimulated the production of MCP-1, IL8, TNFα and IL1β and markedly sensitized the cells to subsequent challenge with inflammatory agents. Thus one explanation could be that statins „pre-stimulate“ the cells leading to an enhanced response to agonists such as cytokines or APLA. The enhanced adhesion molecule response of statin pre-treated cells not necessarily translates into an increased inflammatory response in vivo. Indeed, Bernot et al. (16) observed that statins increased the adhesion molecule response of TNFα-treated EC, but that leukocyte adhesion was decreased due to an inhibition of Rho geranylgeranylation, which block the clustering of adhesion molecules at the EC surface.

The effect of fluvastatin was HMG-CoA-reductase dependent since it was reversed by mevalonate. In addition, the effect of fluvastatin was inhibited by the isoprenoid intermediate GPP and mimicked by the geranylgeranyl-transferase inhibitor GGTL-286, which suggests that the observed effect is due to an inhibition of protein geranylgeranylation. These results are similar to our previous findings that the enhancing effect of simvastatin or fluvastatin on TNFα- or LPS-induced E-selectin expression was reversed by GPP and mimicked by GGTL-286. This indicates that the inhibition of protein geranylgeranylation, but not of cholesterol synthesis, is associated with the effect of these statins (15). More than one hundred small GTP-binding proteins are known that require post-translational geranylgeranylation for their anchoring to the cell membrane, which is essential for their activity. Our data suggest that one or more of these geranylgeranylated proteins exert a negative retrocontrol on EC activation induced by APLA, TNFα or LPS.

In conclusion, our results show that statins potentiate the activation of endothelial cells in response to APLA, leading to an increased expression of leukocyte adhesion molecules, of a monocyte chemoattractant chemokine and of tissue factor. In combination with an increased expression of tissue factor by adherent activated monocytes, these changes alone would be expected to lead to an increased tendency for local thrombus formation. This implies that the well-known clinical benefits of statins are not due to a decrease in the inflammatory response of endothelial cells to cytokines or autoantibodies. In addition, our findings suggest that a geranylgeranylated protein limits the response of endothelial cells to APLA.
References