Role of reactive oxygen species and p38 MAPK in the induction of the pro-adhesive endothelial state mediated by IgG from patients with anti-phospholipid syndrome

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Abstract

The association of the presence of anti-phospholipid antibodies (aPL) with thrombosis characterizes the anti-phospholipid syndrome (APS). The activation of the endothelium is a key event in the establishment of the thrombophilic state. However, the intracellular mechanisms leading to endothelial dysfunction are not fully elucidated. We investigated the role of reactive oxygen species (ROS) in the pro-adhesive state elicited by aPL and studied ROS-dependent downstream signaling pathways. Independent incubation of human umbilical vein endothelial cells (HUVEC) with IgG (IgG-APS) from 12 APS patients caused a large and sustained increase in ROS, which was prevented by the antioxidants vitamin C and N-acetyl-L-cysteine. ROS inhibition observed in the presence of diphenylene iodonium and rotenone indicated an involvement of a membrane-bound oxidase and the mitochondrial transport chain as sources of ROS. ROS acted as a second messenger by activating the p38 mitogen-activated protein kinase and its subsequent target, the stress-related transcription factor activating transcription factor-2 (ATF-2). ROS controlled the up-regulation of vascular cell adhesion molecule-1 expression by IgG-APS-stimulated HUVEC and the increase in THP-1 monocytic cells adhesion. The IgG-APS-mediated oxidative stress was observed irrespective of the clinical and biological criterions of the patients studied here. Taken together, these data indicate that the oxidative stress induced by IgG-APS is a key intracellular event that might contribute to the thrombotic complications of APS by controlling the endothelial adhesive phenotype.

Introduction

The anti-phospholipid syndrome (APS) also called Hughes’s syndrome (1) is an autoimmune disease characterized by the occurrence of venous and/or arterial thrombosis and/or recurrent miscarriage associated with evidence of persistent anti-phospholipid antibodies (aPL). APS, when associated with systemic autoimmune disease, mainly systemic lupus erythematosus (SLE), is classified as secondary. APS without SLE is classified as primary (2, 3). The aPL target anionic phospholipids in the presence of protein co-factors such as β2-glycoprotein I (β2-GPI), annexin V and prothrombin. Among these, β2-GPI is the most extensively studied. The aPL are defined as lupus anticoagulant by their property of prolonging phospholipid-dependent coagulation time and as anti-cardiolipin by their reactivity with the cardiolipin in a solid phase assay (4).

A hyper-coagulable and pro-adhesive state is the main feature of APS patients in vivo. It is characterized by an increase in plasma levels of tissue factor and a decrease of its specific inhibitor tissue factor pathway inhibitor (TFPI), and an enhancement of the release of endothelial microparticles (5, 6). The aPL play a causal role in the development of thrombosis and fetal loss. Mice infused with human aPL...
the induction of adhesion molecules involved in the re-
production of inflammatory cytokines and chemokines, and
including the phosphorylation of transcription factors, the
p38 mediates signals for important biological responses
(13). Once activated by the upstream kinases MKK3/6 (14),
p38 mediates signals for important biological responses
including the phosphorylation of transcription factors, the
production of inflammatory cytokines and chemokines, and
the induction of adhesion molecules involved in the re-
cruitment of leukocytes to the vessel wall (15–17).

Increased expression of adhesion molecules and monocyte
recruitment participates in the ECs dysfunction in APS (8). The
role of aPL in the induction of a pro-adhesive state is clearly
established, however, the precise intracellular mechanisms
are poorly understood. The aim of this work was to study the
intracellular pathways leading to a pro-adhesive state in
human umbilical vein endothelial cells (HUVEC) stimulated by
IgG from APS patients. The aPL-mediated increase in vascular
cell adhesion molecule-1 (VCAM-1) expression and the firm
adhesion of the monocytic cell line THP-1 to HUVEC depen-
ded on the induction of an oxidative stress including ROS
generation and p38 activation. This redox-sensitive pathway
might play a central role in the elicitation of thrombotic events
in the APS.

Methods

Patients

The project was approved by the local institutional ethics
committee (CCPRB no. 02/07) and an informed consent was
received in all cases. Twelve patients with APS (Department of
Internal Medicine, Hôpital de la Conception, Marseille, France)
were studied. They met the Sapporo-revised criteria for APS
(18). Patients (2 males, 44–52 years; 10 females, mean age:
41.1 years; range 22–60 years) have experienced at least one
definite thrombotic event and showed persistent levels of
anti-cardiolipin antibodies and/or lupus anticoagulant. All of
them received anticoagulation treatment (Table 1). Twelve age-
and sex-matched normal healthy volunteers from the
hospital staff (8 females, mean age: 39.8 years; range: 23–55
years; 2 males, 37–46 years) without aPL and thrombosis were
included in this study. Blood samples were collected in the
morning, immediately centrifuged and sera were kept at
−80°C until use.

IgG isolation

Total IgG from sera of APS patients (IgG-APS) or normal
healthy subjects (IgG-NHS) were purified by protein

Table 1. Clinical features of the patients studied

<table>
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<th>Patients</th>
<th>Age (years)</th>
<th>Gender</th>
<th>LAa</th>
<th>IgG a-CLb</th>
<th>IgG a-L2-GPib</th>
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NA: Not applicable.

aLA: Lupus anticoagulant; screening for positivity according to the Sapporo criterion (18).
bAnti-cardiolipin (aCL) and anti-L2-GPI (a-L2-GPI) IgG assayed by an in-house ELISA (54).
cI: Primary APS. II: Secondary APS.
dIgG from patients used for western blotting, VCAM-1 mRNA and microscopically detected fluorescent ROS determinations.
G-Sepharose affinity chromatography (Amersham Biotech, Piscataway, NJ, USA). IgG concentrations were determined by laser nephelometry and purity was checked by SDS-PAGE. Absence of endotoxin was controlled by the Limulus Amebocyte Lysate Endotochome test (Roche Diagnostics, Mannheim, Germany).

**Deprivation of anti-β2-GPI antibodies**

Sera were deprived of anti-β2-GPI antibodies by affinity chromatography. In brief, purified human β2-GPI (kindly given by Diagnostica Stago) was coupled to NHS-Sepharose (Amersham Biotech) as indicated by the manufacturer. Sera of APS patient were applied to the column. The effluent was then passed through a protein G-Sepharose column to isolate total IgG as described above. Absence of anti-β2-GPI antibodies in the IgG fraction was verified by assaying their levels before and after affinity chromatography using the Asserachrom anti-β2-GPI IgG kit (Diagnostica Stago, Asnières, France).

**Cell culture**

HUVEC obtained according to the method of Jaffe et al. (19) were used between the first and third passages. Cells were stimulated by IgG from the different groups of patients or healthy subjects in RPMI 1640 medium supplemented with 20% heat-inactivated FCS. Viability of HUVEC cultured without or with drugs was determined by the incorporation of the fluorescent dye Alamar Blue.

**THP-1 adhesion to HUVEC**

Adhesion of the monocytic cell line THP-1 to HUVEC was performed as described by Akeson et al. (20) using calcein-labeled cells. HUVEC (10,000 cells per well) were stimulated by 200 μg ml⁻¹ of IgG-APS or IgG-NHS for 6 h in 96-well plates in RPMI medium supplemented with 20% FCS. At the end of the stimulation, 500,000 calcein-labeled THP-1 per well were incubated with HUVEC for 30 min in RPMI medium. After washing, THP-1 adhesion was determined by cytofluorimetry using 485 and 530 nm as excitation and emission wavelengths, respectively. Tumor necrosis factor-α (TNF-α) (25 ng ml⁻¹) was used as a positive control and yielded a reproducible 4-fold increase in THP-1 adhesion.

**Intracellular ROS analysis**

Three methods were used to detect intracellular ROS. HUVEC cultured on chamber slides coated with gelatin were stimulated for 2 h with IgG-APS or IgG-NHS and stained with the fluorescent probe dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probes Eugene, OR, USA), 10 μM. Fluorescence was analyzed on an inverted microscope Olympus at an excitation wavelength of 495 nm equipped with a FITC filter. Time response of ROS production was studied in HUVEC plated on 96-well plates. Cells were pre-incubated with 10 μM DCF-DA (21) for 30 min in HBSS, washed and stimulated with IgG-APS or IgG-NHS for different times. Fluorescence was monitored in a plate-reader fluorimeter (Cytofluor 4000, Perseptive Biosystems, Norwalk, CT, USA) using excitation and emission wavelengths of 485 and 530 nm, respectively.

Intracellular ROS were also detected using dihydroadmine-123 (DHR-123, Molecular Probes) (21). HUVEC were stimulated with IgG-APS or IgG-NHS for 2 h, scraped and incubated with DHR-123 (5 μM). The fluorescence was monitored by flow cytometry (Epics XL, Beckman-Coulter, Roissy, France) using excitation and emission wavelengths of 480 and 530 nm, respectively.

When required, HUVEC were pre-incubated 1 h with antioxidants N-acetyl-L-cysteine (NAC) or vitamin C (vit C) and stimulated with IgG-APS or IgG-NHS (200 μg ml⁻¹) in the presence of the drugs. HUVEC were also pre-incubated and stimulated in the presence of different amounts of inhibitors of ROS production, (i) the flavoprotein inhibitor, diphenylene iodonium, (ii) a NO synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME); (iii) a xanthine oxidase inhibitor, allopurinol; or (iv) a mitochondrial inhibitor, rotenone. Twelve independent experiments with IgG-APS or IgG-NHS were performed and each assay was done in triplicate. The viability of the cells was always checked by Alamar Blue incorporation at the end of the incubation with the drugs.

**Western blot and in vitro kinase assay**

Phosphorylations of both p38 MAPK and the transcription factor ATF-2 were analyzed by western blot with rabbit anti-phospho p38, anti-phospho ATF-2 (Cell Signaling, Beverly, MA, USA) and visualized with enhanced chemiluminescent (ECL) reagent. As loading controls, antibodies against the unphosphorylated forms were used. For the in vitro kinase assay, p-ATF-2 was immunoprecipitated from HUVEC lysates and revealed by western blotting using the p38 kinase assay kit (Cell Signaling) and ECL detection.

**VCAM-1 expression**

HUVEC were cultured for 18 h with IgG-APS or IgG-NHS in RPMI medium containing 20% FCS. The expression of VCAM-1 on HUVEC was determined by a cell ELISA (22) using the fluorescent substrate Attophos (Roche Diagnostics). Fluorescence was determined by cytofluorimetry using excitation and emission wavelengths of 450 and 580 nm, respectively. TNF-α (25 ng ml⁻¹) was used as a positive control and yielded a reproducible 13.8-fold increase in VCAM-1 expression.

**Reverse Transcription-PCR**

Total RNA (100 ng) were reverse transcribed and amplified using the Superscript II reverse PCR kit (Invitrogen, Carlsbad, CA, USA) using the following primers for VCAM-1: sense, TTCTTCGGCTGACTCACGACC, and antisense, TCTGTCCAGCTTTGATG; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control: sense, GAGTCAACGGATTGTTCGTGTTTTCGTA, antisense: TTGATTTTGGAGGGATCTCG. Reverse transcription was carried out at 42°C for 30 min. PCR rounds were repeated for 28 cycles with VCAM-1 and 22 cycles with GAPDH giving PCR products of 587 and 238 bp, respectively, for VCAM-1 and GAPDH. Densitometric analysis of the PCR products was carried out using Scion software, and results were expressed as a ratio of change of VCAM-1 versus GAPDH intensities.
Statistical analysis

Values presented are means ± SEM. Data were compared with the use of a two-tailed unpaired Student’s t-test using \( P < 0.5 \) as the level of significance.

Results

Role of IgG-APS on VCAM-1 expression and adhesion of THP-1 cells

We first confirmed that IgG-APS induced a pro-adhesive state in HUVEC. Twelve independent VCAM-1 assays were performed after stimulation of HUVEC with each IgG-APS or each IgG-NHS. IgG-APS elicited a significant dose-dependent increase in membrane-bound VCAM-1 with a 4.1-fold increase in mean for 200 µg ml\(^{-1}\) IgG-APS versus IgG-NHS (\( P < 0.001 \)) in HUVEC (Fig. 1A, left panel) and a time-dependent increase (Fig. 1A, central panel). IgG-APS increased VCAM-1 gene transcription by 4.9-fold in mean with a maximum at 6 h of stimulation (Fig. 1A, right panel). IgG-APS also initiated the adhesion of THP-1 to HUVEC (Fig. 1B) with a 2.8-fold increase in mean for IgG-APS versus IgG-NHS. As a control, stimulation of HUVEC with TNF-α (25 ng ml\(^{-1}\)) induced a 13.8-fold increase of VCAM-1 protein and a 4.5-fold increase in THP-1 adhesion compared with unstimulated cells (data not shown).

Generation of ROS by IgG-APS and inhibition by antioxidants

ROS regulate several classes of genes including adhesion molecules (23), so we investigated whether IgG from APS patients could trigger ROS production in HUVEC. A 2-h stimulation of HUVEC with IgG-APS induced a marked increase in the DCF-DA fluorescent staining of ROS generation.

Fig. 1. Induction of a pro-adhesive state in HUVEC by aPL. (A) Dose- and time-dependent up-regulation of VCAM-1 in HUVEC. HUVEC were stimulated during 18 h with increasing doses of IgG-APS (solid lines with filled squares) or IgG-NHS (dashed lines with filled triangles) from each patient or healthy donor (A, left panel) and for different times with 200 µg ml\(^{-1}\) of IgG (A, central panel). Membrane-bound VCAM-1 was assayed in triplicate wells by a fluorescent cytoELISA. Results were expressed in relative fluorescent unit (RFU). The curves represent the mean values ± SEM from 12 independent experiments. **\( P < 0.01 \), ***\( P < 0.001 \) for IgG-APS versus IgG-NHS at the same concentration or time. In a representative profile of VCAM-1 transcription (A, right panel), the bars indicate mean ± SEM ratios of densitometric scanning of VCAM-1 mRNA versus the corresponding GAPDH profile performed in four independent profiles with patients mentioned in Table 1 or four healthy donors. The ratio of IgG-NHS/GAPDH was arbitrarily defined as 1. ***\( P < 0.001 \) VCAM-1 mRNA by IgG-APS versus IgG-NHS, at 4 and 6 h; **\( P < 0.05 \) VCAM-1 mRNA by IgG-APS at 6 versus 4 h. (B) IgG-APS-mediated THP-1 adhesion THP-1 adhesion to HUVEC was assayed by fluorescent quantifications. The bars represent the mean values ± SEM from 12 independent experiments.
compared with the IgG-NHS (Fig. 2A, a versus e). In 12 independent experiments performed with IgG-APS from each patient, ROS generation gradually increased up to 6 h. This increase was significant as soon as 0.5 h ($P < 0.01$) and reached a 4.8-fold increase in mean ($P < 0.001$) after 3 h of stimulation (Fig. 2B). ROS formation was confirmed by flow cytometry. Incubation of IgG-APS-stimulated HUVEC with the fluorescent probe DHR-123 generated a significant increase ($P < 0.01$) of the mean fluorescence index (Fig. 2C, a and h).

Pre-incubation of HUVEC for 1 h with the antioxidants either vit C (24) or NAC (25) and then stimulation of HUVEC with IgG-APS in the presence of the drugs dose dependently and significantly reduced ROS expression by 38.2 ± 8.6% and 41.1 ± 9.4%, respectively (Fig. 3A, upper and central panels). The inhibitory effect was enhanced when the two drugs were added at the same time during the pre-incubation and the stimulation steps (Fig. 3A, lower panel, 52.9 ± 10.8% inhibition for vit C 100 μM + NAC 5 mM). The use of the antioxidants also resulted in a decrease in fluorescence intensity (Fig. 2A, b, c and d versus a) and in a shift towards lower DHR-123 fluorescence intensities (Fig. 2C, c, e, g and h) similar to those observed with IgG-NHS (Fig. 2C, b, d, f and h).

We then analyzed which oxidative systems may contribute to the production of ROS after IgG-APS exposure of HUVEC. HUVEC were pre-incubated 1 h with the drugs and then stimulated with IgG-APS or IgG-NHS in the presence of identical doses of drugs. Dipherylene iodonium dose dependently inhibited the production of ROS by IgG-APS (41.3 ± 12.5% reduction for 10 μM, $P < 0.01$; Fig. 3B, a). ROS production was partially inhibited in the presence of L-NAME, (27.5 ± 7.8% reduction for 50 μM $P < 0.5$; 3B, b) and by rotenone (23.2 ± 7.9% reduction for 50 μM, $P < 0.5$; 3B, c). Allopurinol did not inhibit the IgG-APS-induced ROS formation (data not shown).

**Effect of anti-β2-GPI deprivation on VCAM-1 up-regulation and ROS formation**

It is well established that anti-β2-GPI antibodies may activate ECs (26). The role of anti-β2-GPI antibodies in the up-regulation of VCAM-1 and ROS formation was investigated. Studies from independent patients indicated that IgG-APS up-regulated VCAM-1 (Fig. 4A, left panel) and induced ROS formation (Fig. 4B, left panel) with some individual variations irrespective of the presence (hatched bars) or the absence (black bars) of anti-β2-GPI antibodies in the IgG fraction. To analyze the relevance of anti-β2-GPI IgG, sera were deprived of anti-β2-GPI antibodies and the IgG fraction was then purified. Stimulation of HUVEC with IgG-APS deprived of anti-β2-GPI slightly reduced the up-regulation of VCAM-1 (Fig. 4A, right panel) or the generation of ROS (Fig. 4B, right panel), but the reduction was not significantly different from the mean increase observed with the non-depleted IgG-APS.

**p38 MAPK activation by IgG-APS and role of ROS**

Among the MAPK members, p38 is sensitive to cellular stress (13). We investigated whether ROS activated the p38 cascade. Stimulation of HUVEC with IgG-APS resulted rapidly in an increase in the phosphorylation of p38, an effect that was maximally achieved by 1 h, and gradually declined. (Fig. 5A, left panel). The p38 phosphorylation observed after a 1 h stimulation with IgG-NHS was similar to that observed in unstimulated cells (Fig. 5A, right panel) and was not modified when the incubation time was prolonged until 18 h (data not shown). IgG-APS also triggered the phosphorylation of the nuclear transcription factor ATF-2 with a maximum at 1.5 h (Fig. 5B). To study the involvement of p38 in the activation of the transcription factor ATF-2, we used inhibitors of p38 activity. Pre-incubation of HUVEC with 10 μM SB203580 (27) and then stimulation of the cells with 200 μg ml⁻¹ IgG-APS in the presence of the inhibitor at the same dose prevented the phosphorylation of ATF-2. As this inhibitor can also inhibit other kinases, we performed similar experiments with 10 μM SB202190, another selective inhibitor of p38 activity (28). SB202190 also prevented ATF-2 phosphorylation (Fig. 5C). These results indicated that the activation of ATF-2 was dependent on the kinase activity of p38.

The involvement of ROS in p38 activation was then investigated. Pre-incubation of HUVEC with 200 μg vit C, 10 mM NAC or vit C together with NAC prevented the IgG-APS-induced p38 phosphorylation (Fig. 5D), indicating that p38 activation was downstream ROS generation.

**Involvement of ROS and p38 on VCAM-1 expression and THP-1 adhesion**

We next determined the involvement of ROS and p38 in the IgG-APS-mediated VCAM-1 expression and THP-1 adhesion. Stimulation of HUVEC with IgG-APS from each patient in the presence of vit C or NAC dose dependently reduced the up-regulation of VCAM-1 protein expression (Fig. 6A and B). The two drugs simultaneously added totally prevented VCAM-1 protein expression and gene transcription (Fig. 6C and F). The up-regulation of VCAM-1 was also prevented by the pre-incubation of HUVEC with the inhibitors of p38, SB203580 or SB202190 (Fig. 6D and E). As shown in Fig. 6(G), THP-1 adhesion to HUVEC was partially reduced from 42% adhesion with IgG-APS without drugs to 38% in the presence of 200 μM vit C, 32% in the presence of 10 mM NAC or 27% with vit C plus NAC. In addition, SB203580 or SB202190 slightly inhibited THP-1 adhesion.

**Discussion**

The aPL from APS patients activate the endothelium to promote pro-coagulant and pro-adhesive states (29). The present study provides new molecular insights into the signaling cascade generated by IgG from APS patients. The triggering of ECs by these IgG results in the production of ROS that function as intracellular second messengers to activate p38 MAPK. ROS control the endothelial pro-adhesive phenotype by regulating the up-regulation of VCAM-1 and the recruitment of monocytes.

The target specificity of aPL from patients with APS is very broad and leads to the recovery of IgG with a large range of diversity (4). To take into account this diversity, the activation of ECs was performed with the polyclonal IgG-APS fraction that represents all the specificities carried by the IgG in the APS. Our data indicate for the first time that, compared with IgG-NHS, the binding of IgG-APS to HUVEC elicited a redox-sensitive signaling pathway that controls the pro-adhesive phenotype of HUVEC. ROS are a heterogeneous family of several molecular forms (10)
that cannot be easily discriminated. Our data indicate that IgG-APS elicited the production of distinct forms of ROS in HUVEC. Indeed, (i) the antioxidants NAC and vit C exert their antioxidant effects by scavenging different forms of ROS and both inhibited ROS production (24, 25). (ii) DCF-DA and DHR-123 fluorescent probes discriminate distinct molecular forms of ROS. DCF-DA oxidation detects mainly, but not exclusively, intracellular hydrogen peroxide produced from the superoxide ion $O_2^-$. 

Fig. 3. Effect of various antioxidants on IgG-APS induced ROS formation. (A) HUVEC were pre-incubated 1 h with increasing doses of the antioxidants vit C, NAC or vit C and NAC together at the same time, washed and then stimulated for 2 h with IgG-APS (filled bar) or IgG-NHS (open bar) in the presence of identical drug concentrations. At the end of the stimulation, fluorescence of the DCF-DA probe was determined as above. Values were the mean of relative fluorescence intensity ± SEM for 12 independent experiments. **P < 0.01, ***P < 0.001 IgG-APS with antioxidant versus IgG-APS without drugs; (B) HUVEC were pre-incubated 1 h with increasing doses of the drugs, washed and then stimulated for 2 h with IgG-APS (filled bar) or IgG-NHS (open bar) in the presence of identical drug concentrations. Values were the mean ± SEM of 12 independent experiments. **P < 0.01, IgG-APS with drugs versus IgG-APS without drugs.
DHR-123 fluorescence is mainly observed in response to peroxynitrite generation (21). The oxidation of the two probes by IgG-APS is in favor of the production of several classes of ROS. (iii) The partial inhibition of ROS by l-NAME confirmed the production of peroxynitrites. In ECs NADPH oxidase system, xanthine oxidase, nitric oxide synthase or the mitochondrial electron transport chain have been shown to participate in the generation of ROS (30). The inhibition of the fluorescence by DPI indicates that a flavin-containing oxidase was involved in the generation of ROS. Although DPI is a major inhibitor of the plasma membrane-associated oxidase NADPH (21), it also inhibits mitochondrial \( \text{O}_2^{\cdot\cdot} \) production by NADH ubiquinone reductase (31). The mitochondrial electron transport chain should also be involved in the IgG-APS-mediated ROS generation as indicated by the partial inhibition induced by rotenone. The flavin-containing enzymes involved in the production of ROS and the upstream events leading to ROS production remain to be characterized. Given the heterogeneity of the cell targets recognized by IgG-APS, it remains possible that several pathways converge to elicit ROS production.

ROS-dependent signaling cascades are mediated by the activation of different kinases (13). In this study, ROS production exerted a prominent function in the activation of a specific signaling cascade. In response to IgG-APS the rapid and sustained phosphorylation of p38 and the activation of its kinase activity allows the subsequent recruitment of ATF-2 as a downstream target. The activation of p38 was redox sensitive as indicated by the inhibition of its phosphorylation

![Fig. 4. Effect of the depletion of anti-\( \beta_2 \)-GPI IgG antibodies on HUVEC activation.](image-url)

The effect of the depletion of anti-\( \beta_2 \)-GPI IgG (a-\( \beta_2 \)-GPI) antibodies in VCAM-1 up-regulation and ROS formation was investigated by the determination of individual variations in VCAM-1 (A, left panel) or ROS formation (B, left panel) obtained with IgG-NHS, open bars; IgG-APS from patients with anti-\( \beta_2 \)-GPI IgG antibodies, hatched bars; IgG-APS from patients without anti-\( \beta_2 \)-GPI IgG antibodies, black bars. VCAM-1 was assayed as described in Fig. 1. ROS formation was determined after a 2-h stimulation of HUVEC by DCF-DA fluorescence. The effect of the deprivation in anti-\( \beta_2 \)-GPI IgG was studied in VCAM-1 up-regulation (A, right panel) or ROS generation (B, right panel) determined in seven independent experiments performed with the seven patients mentioned in the left panels, positive for anti-\( \beta_2 \)-GPI IgG, before (with a-\( \beta_2 \)-GPI) or after deprivation (without a-\( \beta_2 \)-GPI). The bars represent the mean values ± SEM. *** \( P < 0.001 \) IgG-APS versus IgG-NHS; NS: not significant.
by vit C and NAC. ROS regulate several classes of genes including adhesion molecules and transcription factors such as nuclear factor-κB (NF-κB) (32–34). The latter contributes to the aPL-mediated increase in adhesion molecules and tissue factor (35–37). The activation of the transcription factor ATF-2 suggests that other non-NF-κB-signaling mechanism could be involved in the induction of the endothelial adhesive phenotype mediated by IgG-APS. ATF-2 has been shown to physically interact with NF-κB and to participate in the TNF-mediated E-selectin gene transcription (38). The ROS-dependent activation of ATF-2 could contribute to the amplification of the endothelial dysfunction in APS.

The expression of a pro-adhesive phenotype is linked to the endothelial dysfunction in APS (29). Our results provide strong evidence that the adhesive state was directly linked to the production of ROS and the subsequent p38 activation. Inhibiting the rise in ROS levels and the p38 activation totally prevented both VCAM-1 gene transcription and protein up-regulation in response to IgG-APS and partially inhibited the adhesion of THP-1 cells to HUVEC. Two hypotheses could explain this partial inhibition. First, other adhesion molecules known to be up-regulated by aPL (35, 39) could participate in THP-1 adhesion to HUVEC in response to IgG-APS. Second, several signaling pathways could converge to modulate the

![Fig. 5](https://academic.oup.com/intimm/article-abstract/17/4/489/680461)

**Fig. 5.** Effect of ROS on aPL-dependent activation of p38 MAPK. Representative experiments of time-dependent phosphorylation of p38 MAPK and ATF-2 in HUVEC incubated with IgG-APS (200 μg ml⁻¹) for various times. Each cell lysate (5 μg) was resolved on 12% SDS-P under reducing conditions. The blots were probed with (A) anti-p-p38 or (B) anti-p-ATF-2 polyclonal rabbit antibodies to detect the phosphorylated proteins (upper panels). Blots with anti-p38 MAPK or anti-ATF-2 polyclonal antibodies were, respectively, used as loading controls. (C) Representative experiment of an in vitro p38 kinase assay. Immunoprecipitation was performed with anti-p-p38 on cell lysates (200 μg) from HUVEC pre-incubated or not with 10 μM of the inhibitors SB203580 or SB202190. The cells were stimulated 2 h with IgG-NHS or IgG-APS (200 μg ml⁻¹) without or with the drug. Immunoblots were performed with anti-p-p38 or anti-ATF-2. Blots were reprobed with anti-ATF-2. (D) Involvement of ROS in the phosphorylation of p38. Immunoblot against p-p38 was performed on lysates from HUVEC stimulated with IgG-APS or IgG-NHS for 1 h without or with vit C (200 μM), NAC (10 mM), or vit C (100 μM) + NAC (5 mM) at the same time. All the profiles were representative of four independent experiments performed with IgG-APS from the patients indicated in Table 1.
Fig. 6. Involvement of ROS and p38 MAPK in VCAM-1 expression and THP-1 adhesion. (A–E) Dose-dependent inhibition of the up-regulation of VCAM-1 protein by vit C (200 μM), NAC (10 mM), vit C + NAC (100 μM + 5 mM), SB203580 (10 μM) and SB202190 (10 μM). HUVEC were pre-incubated 1 h with increasing doses of drugs and stimulated with IgG-APS or IgG-NHS (200 μg ml⁻¹) in the presence of the drugs for 18 h. VCAM-1 expression was determined as in Fig. 1. (F) Representative pattern of VCAM-1 gene transcription after a 6-h stimulation of HUVEC with IgG-APS or IgG-NHS (200 μg ml⁻¹) in the presence or absence of the drugs. Bars represented the mean ratios ± SEM of densitometric scanning of VCAM-1 mRNA transcription in four independent experiments performed with the patients included in Table 1. (G) Involvement of ROS and p38 on THP-1 adhesion to HUVEC. HUVEC were pre-incubated 1 h with increasing doses of drugs and stimulated with IgG-APS or IgG-NHS (200 μg ml⁻¹) in the presence of the drugs for 6 h. The bars represented mean ± SEM of 12 independent experiments. *P < 0.5, **P < 0.01, ***P < 0.001, IgG-APS with drugs versus IgG-APS without drugs.
pro-adhesive phenotype in APS. Recently, it has been shown that the interaction of anti-β2-GPI antibodies with a Toll-like receptor/IL-1 member mediated the expression of E-selectin by activating a signaling pathway involving TRAF6 and MYD88 (40) and that p38 activation also results from the bridging of β2-GPI by its specific antibodies and the subsequent binding to the apoE-receptor 2 (41). Besides their role in the establishment of an adhesive state, ROS and p38 also participate in the elicitation of the pro-coagulant state observed in APS by controlling the increase of tissue factor expression in monocytes (42).

The aPL form a heterogeneous group of antibodies displaying numerous specificities that could participate in the pathogenic mechanisms of APS (43–45). This heterogeneity is evident in terms of (i) biological activity (4) (presence or absence of LA), (ii) binding to plasma proteins in particular prothrombin, annexin A5 and β2-GPI (46, 47), or (iii) receptors involved in the signaling pathway (48). Some APS patients display auto-antibodies against β2-GPI and their presence has been shown to contribute to the endothelial dysfunction observed in APS (29, 41) by facilitating the binding of β2-GPI to its different receptors on the endothelial membrane. Unexpectedly in our work, anti-β2-GPI antibodies did not contribute for a large part to ROS generation. Indeed, (i) the increase in ROS formation and VCAM-1 up-regulation was comparable in HUVEC stimulated with IgG-APS from patients displaying or not anti-β2-GPI antibodies in their plasma and (ii) in patients with anti-β2-GPI antibodies, the depletion of these antibodies did not significantly modify both ROS generation and VCAM-1 expression. β2-GPI is a protein present in FCS and all the experiments described in this paper were performed in presence of FCS. It remains possible that some β2-GPI-dependent aPL, different from anti-β2-GPI antibodies, bound to the serum β2-GPI and thus contributed to ROS formation.

It is known that among aPL, β2-GPI-dependent and β2-GPI-independent antibodies co-exist, and different studies have shown that these latter antibodies can mediate the effect of aPL. At first, a meta-analysis studying the correlation of thrombotic events with the presence of anti-β2-GPI antibodies did not support a direct role of these antibodies in the elicitation of thrombosis (49). Moreover, in murine experimental models, β2-GPI-dependent as well as β2-GPI-independent antibodies bound to trophoblast membranes and elicited fetal loss or fetal injury by promoting an inflammatory environment (50, 51). So, in our work, it remains possible that β2-GPI-independent antibodies or antibodies requiring this protein as a co-factor activate ECs.

The fact that IgG-APS induced an oxidative stress in vitro is in agreement with the presence in APS patients of an oxidative stress associated with an increased thrombotic risk in vivo. The lipid peroxidation might contribute to the generation of auto-antibodies and the induction of a hyper-coagulable state (52, 53). The oxidative stress elicited by IgG-APS in ECs reflects the pathogenicity of the peroxisative state associated in vivo with aPL in APS patients.

In conclusion, the data obtained in this work indicate that the binding of IgG-APS to their targets initiated an outside–in signaling pathway involving ROS and p38 activation that leads to endothelial dysfunction. Induction of an oxidative stress by IgG-APS represents a new pathway potentially contributing to the thrombo-embolic complications in APS. The knowledge of this mechanism would provide alternative targets for the development of new therapeutic interventions.

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Abbreviations

aPL anti-phospholipid antibodies
APS anti-phospholipid syndrome
DCDF-DA dichlorodihydrofluorescein diacetate
DHR-123 dihydroethidium-123
EC endothelial cell
ECL enhanced chemiluminescent
GAPDH glyceraldehyde-3-phosphate dehydrogenase
β2-GPI β2-glycoprotein I
HUVEC human umbilical vein endothelial cells
L-NAME Nω-nitro-L-arginine methyl ester
MAPK mitogen-activated protein kinase
NAC N-acetyl-L-cysteine
NF-κB nuclear factor-κB
ROS reactive oxygen species
SLE systemic lupus erythematosus
TNF tumor necrosis factor
VCAM-1 vascular cell adhesion molecule-1
vit C vitamin C

References

Redox and endothelial adhesive phenotype in APS


