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Inhibition of LPS-induced chemokine production in human lung endothelial cells by lipid conjugates anchored to the membrane

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1 In acute respiratory distress syndrome (ARDS) induced by endotoxins, a high production of inflammatory mediators by microvascular lung endothelial cells (LMVEC) can be observed. Activation of cells by endotoxins may result in elevated secretion of phospholipase A_2 ($sPLA_2$) which is thought to contribute to tissue damage. The present study was undertaken to investigate the role of $sPLA_2$ in chemokine production in human lung microvascular endothelial cells (LMVEC) stimulated with the endotoxins lipopolysaccharide (LPS) and lipoteichoic acid (LTA). In particular, we investigated the effects of $sPLA_2$ inhibitors, specifically, the extracellular PLA_2 inhibitors (ExPLIs), composed of N-derivatized phosphatidyl-ethanolamine linked to polymeric carriers, and LY311727, a specific inhibitor of non-pancreatic $sPLA_2$.

2 ExPLIs markedly inhibited LPS and LTA induced production and mRNA expression of the neutrophile attracting chemokines IL-8, Gro-α and ENA-78, as well as of the adhesion molecules ICAM-1 and E-selectin. Concomitantly, ExPLIs inhibited the LPS-induced activation of NF-κB by LPS but not its activation by TNF-α or IL-1.

3 Endotoxin mediated chemokine production in LMVEC seems not to involve PLA_2 activity, since LPS stimulation was not associated with activation of intracellular or secreted PLA_2. It therefore seems that the inhibitory effect of the ExPLIs was not due to their PLA_2 inhibiting capacity. This was supported by the finding that the LPS-induced chemokine production was not affected by the selective $sPLA_2$ inhibitor LY311727.

4 It is proposed that the ExPLIs may be considered a prototype of potent suppressors of specific endotoxin-induced inflammatory responses, with potential implications for the therapy of subsequent severe inflammation.

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Keywords: Acute respiratory distress syndrome; microvascular lung endothelium; lipopolysaccharide; lipoteichonic acid; CXC-chemokines; adhesion molecules; phospholipase A_2; lipid conjugates; extracellular phospholipase inhibitor

Abbreviations: AA, arachidonic acid; ARDS, acute respiratory distress syndrome; AsPE, aspirin and phosphatidylethanolamine; CMPE, carboxymethylcellulose and phosphatidylethanolamine; $cPLA_2$, cytosolic phospholipase A_2; ENA-78, neutrophil activating protein-78; ExPLI, extracellular phospholipase inhibitor; GAG, glucosaminoglycan; Gro-α, growth-related gene α; HyPE, hyaluronic acid and phosphatidylethanolamine; ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin-1; IL-8, interleukin-8; $iPLA_2$, calcium-independent phospholipase A_2; LMVEC, lung microvascular endothelial cell; LPS, lipopolysaccharide; LTA, lipoteichonic acid; NF-κB, nuclear factor-kappa B; OA, oleic acid; PAF, platelet activating factor; PE, phosphatidylethanolamine; PL, phospholipid; PMN, polymorphonuclear leukocytes; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; sPLA2, secretory phospholipase A_2; TNF-α, tumour necrosis factor-alpha; TLR, toll-like-receptor

Introduction

Acute respiratory distress syndrome (ARDS) is a clinically and pathophysiologically complex syndrome of acute lung inflammation. Various injurious and noxious substances may initiate ARDS, but the most frequent causes with severe inflammation are due to bacterial toxins like lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (Middelveld & Alving, 2000).

The pathogenesis of ARDS, like other forms of endotoxin-induced inflammation, involves multiple inflammatory mediators. Among them, secreted phospholipase A_2 ($sPLA_2$) has been proposed to play a major role (Anderson *et al*., 1994). Reportedly, increased levels and activity of $sPLA_2$ in bronchoalveolar lavage fluid (Kim *et al*., 1995) and lung tissue (Ishizaki *et al*., 1989; Nakano & Arita, 1990) were found in patients with ARDS. These high levels of $sPLA_2$ may cause lung injury by damaging alveolar epithelial cells, destroying pulmonary surfactant, promoting platelet-activating factor (PAF) and enhancing the production of eicosanoids that mediate direct or neutrophil-mediated tissue injury

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(Schaefers *et al*., 1996; Holm *et al*., 1991). Furthermore it has been reported, that $sPLA_2$ regulates cytokine production and generation of reactive oxygen species (Pruzanski & Vadas, 1991).

PLA_2 hydrolyzes membrane phospholipids (PL) at the sn-2 position, releasing free fatty acids and phospholipids. The PLA_2s include the intracellular enzymes, specifically the cytosolic ($cPLA_2$) and calcium-independent ($iPLA_2$) ones, and the subfamily of secreted enzymes ($sPLA_2$) (Six & Dennis, 2000). $cPLA_2$ has been reported to play an important role in the development of ARDS, as it is specific to PL that carry arachidonic acid, the precursor of pro-inflammatory eicosanoids (Nagase *et al*., 2000). Yet, under inflammatory conditions, the majority of AA is produced by $sPLA_2$ (Nevalainen *et al*., 2000). In addition to producing lipid mediators, $sPLA_2$ secreted into the extracellular fluid synergizes with other pro-inflammatory mediators in tissue damage occurring at inflammatory sites (Dan *et al*., 1996; Ginsburg *et al*., 1997). It has been suggested that degradation of cell surface glycosaminoglycans (GAG) by reactive oxygen species (Dan *et al*., 1996) and heparinase (Kim *et al*., 2001) render the cell membrane accessible to lysis by exogenous $sPLA_2$. Recently, the existence of distinct $sPLA_2$-subtypes was defined in human lung under septic conditions. The $sPLA_2$-IB, primarily isolated from pancreas, was detectable during lung inflammation as a complication of an acute pancreatitis (Tsukahara *et al*., 1999). The $sPLA_2$-IIA, produced by different cell types, e.g. smooth muscle cells and epithelial cell (Pfeilschifter *et al*., 1989) and isolated from inflammatory fluids, was found to be highly upregulated in bronchoalveolar lavage-fluid (Samet *et al*., 1996). Manifestations of $sPLA_2$-associated lung dysfunction, mainly vasodilation and vascular permeability, have been proposed to be mediated either *via* direct action of $sPLA_2$ itself (Vadas & Pruzanski, 1986) or derivatives of its action, such as eicosanoids and platelet-activating-factor (Demling, 1993). In addition to these well characterized $sPLA_2$ isoenzymes, mRNA expression of $sPLA_2$-V and X was recently detected in mammalian lungs, but their functions in inflammatory processes are still unclear (Seeds *et al*., 2000).

Characteristic for endotoxin-induced cellular responses in the lung are the extravasation and accumulation of activated neutrophils (PMN). PMN adhere to the endothelium *via* adhesion molecules and through endothelial-derived chemokines they migrate along a chemotactic gradient into the inflammed tissue. In lung microvascular endothelial cells members of the CXC-chemokine family IL-8, Gro-α and ENA-78 exhibit strong chemotactic activities to PMN and are strongly upregulated after LPS stimulation (Beck *et al*., 1999). Furthermore, the EC adhesion molecules ICAM-1 and E-selectin, which are involved in mediating PMN adherence, are elevated under endotoxin stimulation (Jersmann *et al*., 2001).

Cell stimulation by endotoxins has been reported to involve activation of $sPLA_2$ in several cell types, such as epithelial cells or astrocytes (Grossman *et al*., 2000; Wang & Sun, 2001). In order to gain insight into the role of endothelial $sPLA_2$ in the pathophysiology of ARDS, we explored the involvement of $sPLA_2$ in LPS-induced production of pro-inflammatory chemokines by human LMVEC, and the possibility of controlling this process by inhibition of $sPLA_2$ activity. To this end, we employed extracellular PLA_2 inhibitors (ExPLIs), designed and synthesized in the laboratory of S. Yedgar. These are composed of lipidic inhibitors of PLA_2, e.g. N-derivatized phosphatidyl-ethanolamine (PE), linked to polymeric carriers, such as hyaluronic acid (Dan *et al*., 1998; Yedgar *et al*., 2000). While the lipid moiety incorporate into the cell membrane, the polymeric carrier prevents its internalization. By combining a GAG-mimicking polymer anchored to the cell membrane by the PLA_2 inhibiting lipid, the ExPLIs might exhibit a dual effect in protecting cells from PLA_2 and pro-inflammatory mediators. Accordingly, they have been found effective in blocking the action of different exogenous PLA_2s (Dan *et al*., 1998). Moreover, they suppress the activation of endogenous PLA_2 induced by ROS in epithelial cells (Yedgar *et al*., 1995; Ginsburg *et al*., 1997) or LPS in macrophages (Balsinde *et al*., 2000), and protect cells from damage induced by oxidizing agents and hemolysins (Yedgar *et al*., 1995, 2000; Ginsburg *et al*., 1997). In previous studies ExPLIs were found to be effective in ameliorating delayed type hypersensitivity in mice (Yedgar *et al*., 1994), and bleomycin-induced lung injury in hamsters (Breuer *et al*., 1995). On these grounds, in the present study we have employed the ExPLIs and LY-311727, a specific $sPLA_2$ inhibitor (Schevitz *et al*., 1995) to explore the role of $sPLA_2$ in endotoxin-induced production of chemokines and adhesion molecules by LMVEC.

Methods

Materials

Essential growth medium for microvascular cells (EGM-MV) and Trypsin 0.025 Vol%/EDTA 0.01 Vol% (T/E) were from CellSystems, Remagen, Germany. Antibodies against ICAM-1 and E-selectin were purchased from DAKO, Glostrup, Denmark. LPS, LTA, TNF-α, IL-1 and hyaluronic acid were from Sigma, Deisenhofen, Germany. IL-8, Gro-α and ENA-78 Immunoassays were obtained from R&D Systems GmbH, Wiesbaden, Germany. The primers of the chemokines IL-8, Gro-α and ENA-78 were designed by Perkin Elmer applied Biosystems, Weiterstadt, Germany. Trizol-Reagent, DNAse and dNTPs were from Gibco BRL, Eggenstein Germany. SuperScript TM II Pre-amplification System was purchased from Life Technologies, Karlsruhe, Germany. Gel shift assay system was from Promega Corporation, U.S.A. BSA and Light Cycler-FastStart DNA Master SYBR GreenI ready-to-use 'Hot start' reaction mix were from Roche, Mannheim, Germany. ^{3}H-Arachidonic acid and ^{3}H-oleic acid were from Amersham Pharmacia Biotech, U.K. 1-^{14}C-oleic acid-labelled *Escherichia coli* membranes were synthesized as described previously (Scholz *et al*., 1999). LY311727 was a generous gift from Eli Lilly Company Greenfield, Indiana, U.S.A. Anti TLR-4 m-ab was from Biocarta, San Diego, California, U.S.A.

Extracellular PLA_2 inhibitors (ExPLIs) were synthesized in the laboratory of S. Yedgar. The ExPLIs used in the present study are composed of N-derivatized dipalmitoyl-phosphatidylethanolamine (PE) linked to one of the following compounds: truncated hyaluronic acid (HyPE); chondroitin sulphate A (CSAPE); carboxymethyl-methylcellulose (CMPE); or aspirin (AsPE). All these compounds were free of endotoxins demonstrated in Limulus amebocyte lysate (Bio Whittaker Comp., Walkersville, U.S.A.).

Cell culture

Human lung microvascular endothelial cells (LMVEC) were purchased from CellSystems, Remagen, Germany at passage 4. The cells were seeded in a density of 5000 cells cm^{-2} in T25 flasks and maintained according to the manufacturer's specification in EGM-MV. After confluency they were subcultured as described previously (Beck *et al*., 1999).

Characterization of the LMVEC was performed on the basis of a positive staining for uptake of acetylated LDL, Factor VIII related antigen and PECAM (CD31) expression as well as negative staining for alpha smooth muscle actin.

In each experiment the viability of LPS- and LTA-stimulated or HyPE treated LMVEC was tested by Trypan blue exclusion.

Chemokine production

LMVEC were seeded in 24-well plates and grown to confluence. The cells were stimulated for 24 h with medium as control or with LPS (*Escherichia coli* (1 $\mu g\ ml^{-1}$)), LTA (*Staphylococcus aureus* (10 $\mu g\ ml^{-1}$)), IL-1 (1 $ng\ ml^{-1}$) or TNF-α (500 $U\ ml^{-1}$) in the presence or absence of HyPE (0.1–20 μM). Alternatively cells were pretreated with LPS or LTA several hours before HYPE was added. Supernatants were collected and the production of ENA-78, Gro-α and IL-8 was measured.

All ELISAs were performed according to the manufacturer's instructions.

RNA isolation and polymerase chain reaction

RT–PCR: Confluent LMVEC were stimulated with medium as control or with LPS (1 $\mu g\ ml^{-1}$) or LTA (10 $\mu g\ ml^{-1}$) in the presence or absence of HyPE (10 μM). Total RNA was isolated using Trizol-Reagent according to the manufacturer's instructions. Each RNA preparation was subjected to DNAse digestion to remove possible contaminations of genomic DNA. Total RNA, (1 μg) was reverse transcribed using SuperScript TM II Pre-amplification System according to the manufacturer's instructions. The oligonucleotides (primers) used for PCR are listed in Table 1.

Amplification of 0.5 μl of cDNA was performed in a total volume of 25 μl containing 19.6 pmol of each chemokine primer: (mM) dNTPs 5, 2.5 U Taq Polymerase, Tris HCl 10, KCl 7.5, $MgCl_2$ 1.5. PCR reactions were initiated at 94°C for 3 min, followed by 30 cycles of amplification, each consisting of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min. At the end of the amplification cycles the products were incubated for 10 min at 72°C. Control samples were constructed either by omitting cDNA synthesis or without addition of cDNA. PCR products were separated on a 1% agarose gel.

Table 1 Primer sequences and base pairs (bp) of the amplicon

Primer	*Sequence*	*Size of PCR product* (bp)
GAPDH	5′-GTCTTCACCACCATGGAGAA-3′ 5′-ATCCACAGTCTTCTGGGTGG-3	268
IL-8	5′-CGATGTCAGTGCATAAAGACA-3′ 5′-TGAATCTCAGCCCTCTTCAAAAA-3′	200
GROα	5′-TGCTCCTGCTCCTGGTAG-3′ 5′-TCCCTTCTGGTCAGTTGG-3′	273
ENA-78	5′CGCTCTCTTGACCACTATGAG-3′ 5′-GCCTATGGCGAACACTTG-3′	232
ICAM-1	5′-CAAGAGGAAGGAGCAAGACTC-3′ 5′-TTGGGGTCAGTAGACAGCAG-3′	263
E-selectin	5′-GCTGTGAGATGCGATGCTGTC-3′ 5′CCTCTAGTTCCCCAGATGCACC-3′	280

Real-time PCR: 500 ng of total RNA of each sample was in addition reverse transcribed into cDNA for Real-time PCR analysis using 1st Strand cDNA Synthesis Kit according to the manufacturer's instructions (Roche). cDNA was diluted in 20 μl DEPC-treated water. DNA standards were generated by PCR amplification of gene products, purification and quantification by spectrophotometry. Real time PCR of cDNA specimens and DNA standards were performed in a total volume of 25 μl in the presence of 2 μl Light cycler–FastStart DNA Master SYBR GreenI reaction mix, 0.5 μM of gen-specific primers and 4 mM $MgCl_2$. Standard curves were generated for all chemokines. PCR efficiency was assessed from the slopes of the standard curves and was found to be between 90 and 100%. Concentration of chemokine cDNA was calculated by linear regression analysis of all standard curves and was corrected for an equal expression of GAPDH. At least five experiments with similar results were performed.

Analysis by fluorescence-activated cell sorter (FACS)

Confluent LMVEC were stimulated with medium as control or with LPS (1 $\mu g\ ml^{-1}$) or LTA (10 $\mu g\ ml^{-1}$) in the presence or absence of HyPE (10 μM). Thereafter cells were harvested by T/E, extensively washed, and monoclonal antibodies directed against the endothelial adhesion molecules ICAM-1 and E-selectin in dilutions of 1 : 20 were added for 30 min at 4°C. In addition unstimulated or stimulated cells were harvested as described and preincubated for 20 min with HyPE (10 μM) and monoclonal antibodies against TLR4. Cells were washed and incubated with an anti-mouse F(ab′)2, FITC conjugated secondary antibody. After washing cells were analysed by FACS-scan.

Electrophorese mobility shift assay (EMSA)

Confluent LMVEC were pre-incubated over night in basal medium containing 0.01% BSA. Hereafter they were stimulated or not for different time periods with LPS, IL-1 or TNF-α in the presence or absence of HyPE. Nuclear extracts were prepared as described previously (Hilschermann *et al*., 1996). Oligonucleotides containing a NF-κB consensus sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) were labelled to a specific activity $>5\times107$ c.p.m. μg^{-1} DNA. NF-κB-binding was performed in (mM) HEPES 10, (pH = 7.5), EDTA 0.5, KCl 70, DTT 2, 2% glycerol, 0.025% NP-40, 4% Ficoll, 0.1 M PMSF, 1 $mg\ ml^{-1}$ BSA and 0.1 $\mu g\ \mu l^{-1}$ poly di/dc in a total volume of 20 μl. Nuclear extracts (10 μg) were incubated for 30 min at room temperature in the presence of 1 ng labelled oligonucleotide. DNA-protein complexes were resolved on 5% non-denaturating polyacrylamide gels electrophoresed in low ionic strength buffer and visualized by autoradiography. Specifity of shifted bands were demonstrated by adding a cold NF-κB consensus sequence or by supershift using anti-p65 antibodies.

Determination of the secretion of $sPLA_2$-activity

Secretion of $sPLA_2$-activity to the culture medium by LMVEC was determined using *Escherichia coli* membranes labelled with 1-^{14}C-oleate as a substrate, as described previously (Scholz *et al*., 1999). Confluent LMVEC were incubated in control medium or stimulated with LPS (1 – 5 μg ml^{-1}) in the absence or presence of HyPE (10 μM) for 24 h, when the culture medium was collected. Since $sPLA_2$ may bind to cell surface proteoglycans, this was done following the addition of heparin to the culture prior to collecting the culture medium, in order to detach $sPLA_2$ from the cell surface (Suga *et al*., 1993). $sPLA_2$ activity in the collected medium was measured as follows: The assay mixtures (750 μl) contained 100 mM Tris/Cl pH = 8.0, 100 mM $CaCl_2$, 0.1% BSA, with (1-^{14}C-oleic acid-labelled *Escherichia coli*, 5000 c.p.m.) and the enzyme-containing culture. The reaction mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition of 50 μl 1 mM EGTA/1 N HCl and 800 μl ethyl acetate. The lipid-containing phase was collected and dried in a vacuum concentrator. The dry lipids were dissolved in 50 μl ethyl acetate and separated by thin layer chromatography on silica gel G 60 plates using the organic phase of ethyl acetate : isooctane : acetic acid : water (110 : 50 : 20 : 100 by volume) as a solvent system. Detection and quantification of the released fatty acid was performed with a Linear Analyzer (Bertold, Wildbad, Germany). In parallel experiments the extraction efficiency was found to be greater than 95%.

Determination of ^{3}H-arachidonic (AA) -and ^{3}H-oleic acid (OA)-release

LMVEC were grown in 12-well plates until confluency. Thereafter they were washed twice with serum-free EBM and incubated with 0.2 μCi/ml of [^{3}H]-AA and [^{3}H]-OA in 1 ml medium overnight at 37°C. Five probes were thereafter solubilized with 600 μl NaOH (1 M), and the radioactivity associated with the cells was measured with a liquid scintillation counter. About 50% of the total radioactivity added was incorporated into the cells. Thereafter the incubation was continued with the other cells without [^{3}H]-AA and [^{3}H]-OA in the absence or presence of LPS (1 μg ml^{-1}) with or without HYPE (10 μM) or palmitoyl-lysophosphatidylcholine- (LPC) (50 μM) for 24 h. Supernatants were collected and counted. The values of the released radioactivity were then expressed as per cent of the total radioactivity incorporated into the cells before the addition of LPS or LPS with HyPE or LPC.

Statistical analysis

Statistical analysis was performed using Stata Statistical software (Mann – Whitney test). A P-value of $P<0.05$ was considered as significant.

Results

Effects of HYPE on the production of chemokines and expression of adhesion molecules in LPS-stimulated LMVEC

It has previously been shown that under basal conditions LMVEC have a low expression of IL-8, ENA-78 and Gro-α, which is strongly upregulated under stimulatory conditions (Beck *et al*., 1999). Since it has been suggested that the action of LPS involves activation of $sPLA_2$, the present study was conducted to examine $sPLA_2$ involvement in LPS-induced chemokine production in LMVEC. To this end we made use of an ExPLI, i.e. HyPE, that effectively inhibits $sPLA_2$ *in vitro* and *in vivo* (Dan *et al*., 1998). While HyPE did not influence the basal chemokine production (not shown), it dose-dependently inhibited LPS-mediated production of all chemokines tested (Figure 1A). Accordingly, HyPE suppressed chemokine mRNA expression (Figure 1B).

Like LPS, LTA is currently considered to be an inducer of inflammatory response in endothelial cells (Kawamura *et al*., 1995), although its influence on $sPLA_2$ activity has not yet been defined. We therefore investigated whether HyPE also inhibits LTA-induced chemokine production in LMVEC. As shown in Figure 2, LTA strongly upregulated the production of IL-8, although to a lesser extent than LPS. Again it was found that HyPE suppressed this production in a dose-dependent manner. The same results were obtained for ENA-78 and Gro-α (data not shown). Whereas a significant upregulation in LPS-mediated chemokine production was observed already after 6 h of stimulation (Figure 3A), upregulation hereof by LTA occured much later (Figure 3B). When HyPE was added to the cell culture in the presence of either stimulus, the upregulation was not observed even after prolonged stimulation.

Since extravasation and migration of neutrophils to inflammed lung tissue is initiated by adherence to the EC surface *via* adhesion molecules, we examined if HyPE was also able to inhibit the expression of ICAM-1 and E-selectin in LPS-stimulated LMVEC. Both ICAM-1 and E-selectin were expressed at a low level under unstimulated conditions and were highly upregulated by stimulation with LPS. Similar to its effect on chemokines, HyPE inhibited the upregulation of mRNA-expression and protein synthesis of these molecules (Figure 4A,B).

Activation of NF-κB by LPS is inhibited by HyPE

It is generally accepted that LPS-induced production of chemokines and adhesion molecules is dependent on activation of the transcription factor NF-κB (Oitzinger *et al*., 2001). Therefore, the influence of HYPE on LPS-induced activation of NF-κB in LMVEC was investigated by performing EMSA. Maximal activation of NF-κB was found between 6 and 10 h after stimulation with LPS. In concordance to its effect on the expression of chemokines and adhesion molecules, HyPE suppressed LPS-induced activation of NF-κB in LMVEC (Figure 5). TNF-α induced NF-κB-activation could not be prevented by HyPE (data not shown).

Role of PLA_2 in LPS-induced chemokine production

In endotoxin-infected lungs, $sPLA_2$ is found to be highly upregulated, and is believed to play an important role in the pathophysiology. In accordance with this, LPS has been found to activate $sPLA_2$ in different cells (Doerfler *et al*., 1994; Fouda *et al*., 1995), however data on direct activation of $sPLA_2$ in LPS stimulated endothelial cells are lacking (Flynn & Hoff, 1995). Therefore we examined the LPS effect on secretion of $sPLA_2$ by

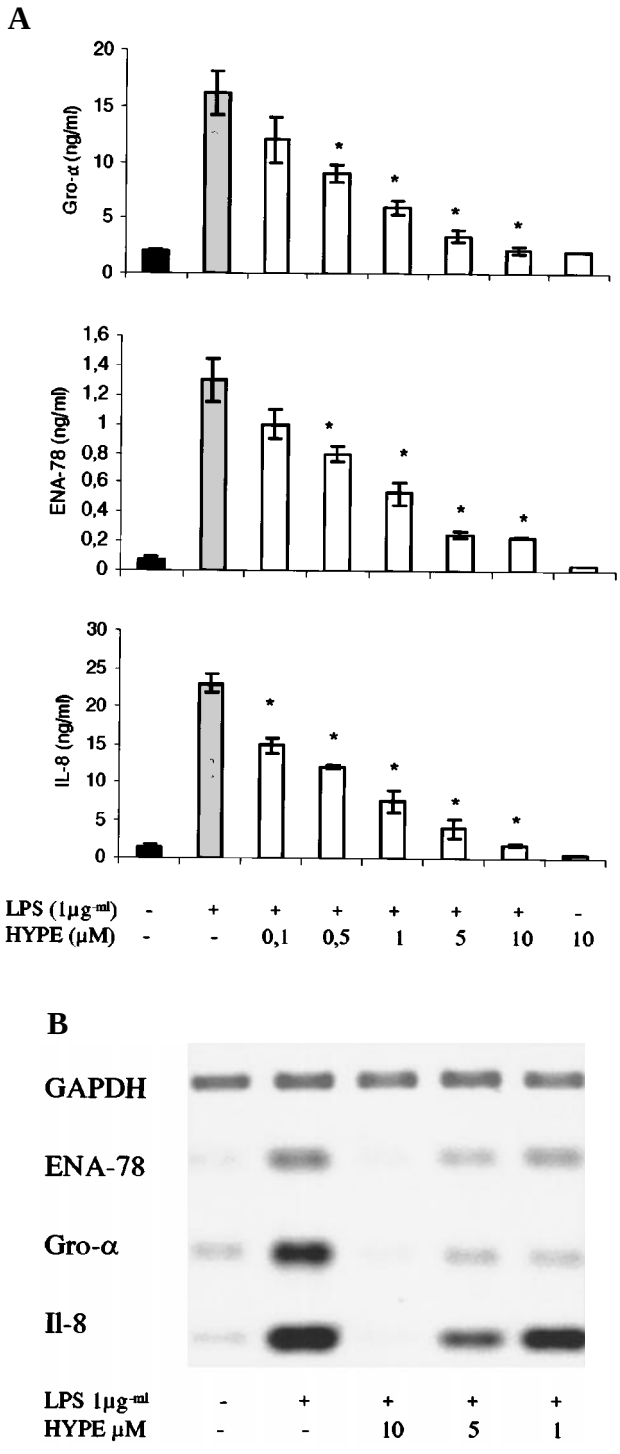

Figure 1 Effect of HyPE on LPS-induced chemokine production. (A) LMVEC were either incubated in control medium (black bar) or stimulated for 24 h with LPS (1 μg ml^{-1}) in the presence (open bar) or absence of HyPE at the indicated concentrations (grey bars). Thereafter, the supernatants were collected and assessed for chemokine production by ELISA. Each value represents the mean production of triplicate cultures $\pm$ s.d.. $^*P<0.05$ compared to cells that were stimulated only with LPS. (B) LMVEC were treated as in (A) and harvested using Trizol-Reagent. Total RNA was isolated and RT–PCR was performed as described in Methods. Five experiments with similar results were performed. The results of one represenative experiment is shown.

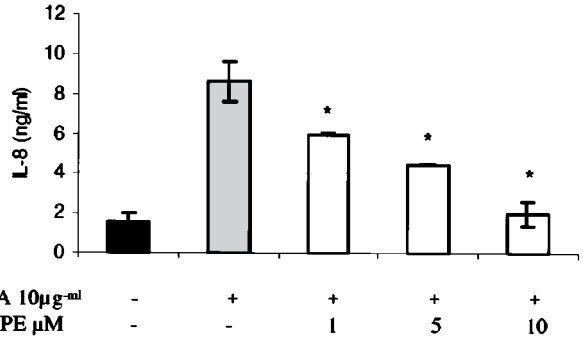

Figure 2 Effect of HyPE on LTA-induced IL-8 production: LMVEC were either unstimulated (black bar) or stimulated with 10 μg ml^{-1} of LTA in the absence (grey bar) or presence of different concentrations of HyPE (open bars). The results of one representative experiment ($n=4$) are expressed as mean IL-8 production $\pm$ s.d. $^*P<0.05$ compared to cells that were stimulated only with LTA.

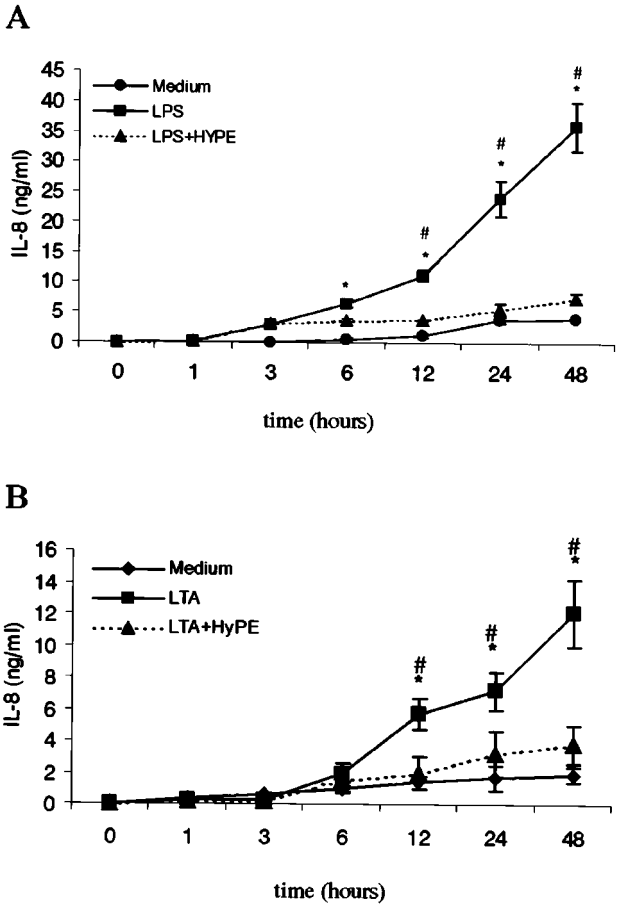

Figure 3 Kinetics of IL-8 production in LPS or LTA treated LMVEC: LMVEC were either unstimulated (circles) or stimulated with 1 μg ml^{-1} LPS (A) or 10 μg ml^{-1} LTA (B) in the absence (squares) or presence of 10 μM HyPE (triangles) for the indicated time periods. The chemokine level in the culture medium was determined by ELISA). The results of one representative experiment ($n=4$) are expressed as mean IL-8 production $\pm$ s.d., $^*P<0.05$ for stimulated and unstimulated cells. $\#P<0.05$ for stimulated LMVEC in the absence or presence of HyPE.

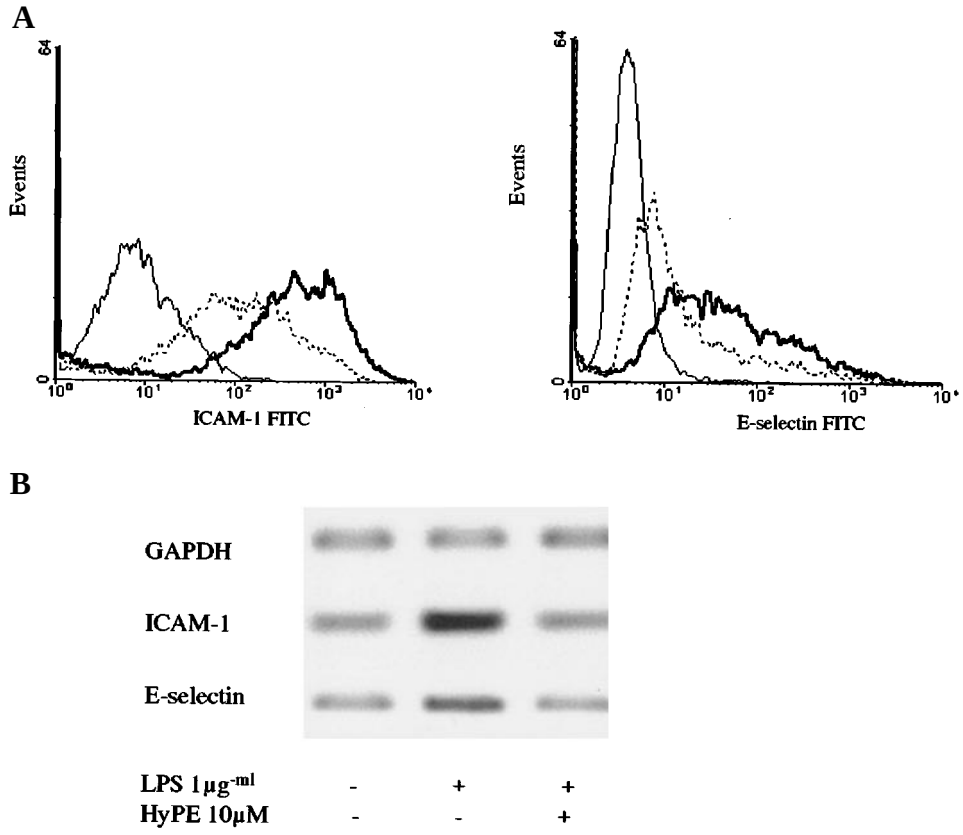

Figure 4 Effect of HyPE on LPS-induced ICAM-1 and E-selectin expression. (A) LMVEC were unstimulated (thin line) or stimulated with LPS (1 μg ml^{-1}) in the absence (bold line) or presence (dotted line) of HyPE (5 mM), then harvested and incubated with the appropriate antibodies for FACS-analysis as described in Methods. (B) LMVEC, treated as in (A), were harvested using Trizol-Reagent. Total RNA was isolated and RT–PCR was performed as described in Methods. Five experiments with similar results were performed. The results of one representative experiment is shown.

LMVEC by determining its activity in the extracellular medium subsequent to their stimulation with LPS, IL-1, TNF-α or combinations thereof. No elevation of $sPLA_2$-activity in the cell culture medium was detected following treatment with LPS and the other stimulators (data not shown), suggesting that $sPLA_2$ is not involved in the LPS-induced endothelial chemokine production. In accordance with this it was found that LY311237 – a known inhibitor of $sPLA_2$ IIA, V and X (Schadow *et al*., 2001), but not of IB or $cPLA_2$ (Schevitz *et al*., 1995) – did not affect LPS-induced chemokine production by LMVEC (Figure 6).

Subsequent to the finding that activation of LMVEC by LPS does not involve secretion of $sPLA_2$, we examined whether PLA_2 activity is involved in this process at all, by utilizing the specificity of $cPLA_2$ to arachidonic acid-containing phospholipids. To this end, phospholipids of LMVEC were metabolically labelled with ^{3}H-AA or OA, and the release of free fatty acids was monitored subsequent to stimulation with LPS. As shown in Figure 7, no increase in the release of either of these fatty acids was observed after LPS stimulation, suggesting that PLA_2 activation is not involved in LPS-induced chemokine production by LMVEC.

Mechanism of HyPE action

As described above, HyPE is an amphipatic complex, which is anchored to the cell membrane by its lipophilic moiety (PE), whereas hyaluronic acid resides at the cell surface. It could thus be argued that the inhibitory effect of HyPE was due to steric hindrance of the polymeric carrier interfering with the interaction of LPS with its receptor at the cell surface. To examine this possibility, we determined the effect of HyPE when it is added to the cell culture after LPS. Figure 8 shows that in this model the inhibition of IL-8 production by HyPE was inversely proportional to the time at which HyPE was added after LPS. This could suggest that HyPE interferes with the signalling downstream after LPS by interaction with its receptor. However, Figure 8 also depicts an experiment in which LPS-containing medium was replaced with LPS-free medium at different times post-stimulation. The results of this experiment show that this treatment was comparable with the addition of HyPE; the reduction in IL-8 production was inversely proportional to the time at which the LPS was washed away. Taken together, these results still leave open the possibility that HyPE, *via* its extracellular polymeric moiety, interfere with the LPS interaction with its receptor.

To further examine this possibility, we performed two kinds of experiments: Firstly, it was tested whether HyPE prevents the binding of antibodies against the transmembrane LPS-receptor TLR4. Figure 9 shows that in the presence of HyPE, LPS–TLR4 interaction was partly inhibited, while under the same conditions, IL-8 production was completely blocked. This seems to support the notion that at least part of the HyPE inhibitory effect is due to interference with LPS

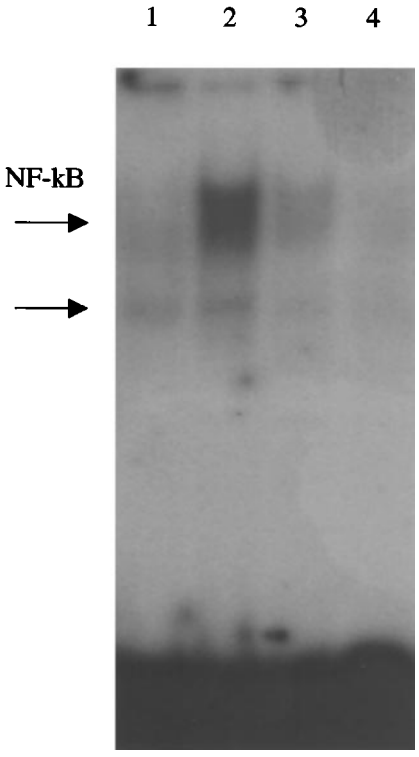

Figure 5 Effect of HyPE on LPS-induced activation of NF-κB in LMVEC. LMVEC were either unstimulated (lane 1) or stimulated for 8 h with LPS in the absence (lane 2) or presence of HYPE (10 μM) (lane 3). To demonstrate specificity of the shifted band, cold NF-κB consensus oligonucleotide were added to nuclear extracts from LPS stimulated cells 30 min prior to addition of the labelled oligonucleotide (lane 4).

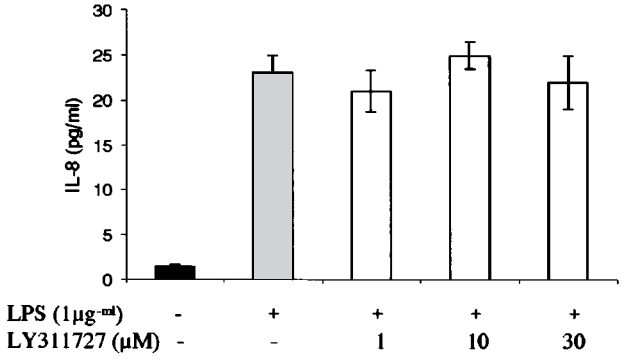

Figure 6 Effect of the sPLA2-inhibitor LY 311727 on LPS-induced IL-8 production. LMVEC were either cultured for 24 h in medium (black bar) or stimulated with LPS (1 μg ml^{-1}) in the presence (open bar) or absence of LY 311727 (grey bars). The culture medium was then collected and assessed for IL-8 production by ELISA. The results of one representative experiment ($n=3$) are expressed as mean IL-8 production ± s.d.

interaction with its receptor. However, in another experiment we employed different ExPLIs, in which the PE moiety was coupled either to polymers that differ in their chemical nature – specifically chondroitin sulphate A (CSAPE) and carboxymethylcellulose (CMPE) – or aspirin (ASPE), as a non polymer-containing inhibitor. It was obtained that all of these inhibitors suppressed LPS-induced chemokine production, while the polymers or aspirin alone were ineffective (data not shown). In addition, HyPE did not inhibit chemokine

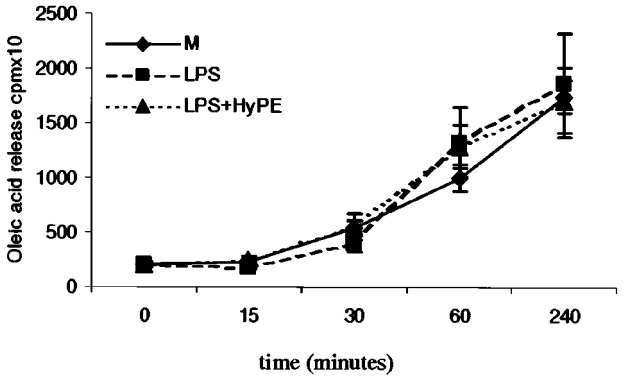

Figure 7 Effect of LPS on sPLA$_2$ activity. Confluent LMVEC were incubated overnight with 0.2 μCi ml^{-1} of [^{3}H]-OA, thereafter extensively washed. Cells were then either unstimulated (circles) or stimulated with LPS (1 μg ml^{-1}) in the absence (squares) or presence (triangle) of HyPE (10 μM). The culture medium was collected at the indicated time and the amount of labelled fatty acid released in the supernatant was determined in a scintillation counter. The results of one representative experiment ($n=3$) are expressed as mean sPLA$_2$ activity ± s.d.

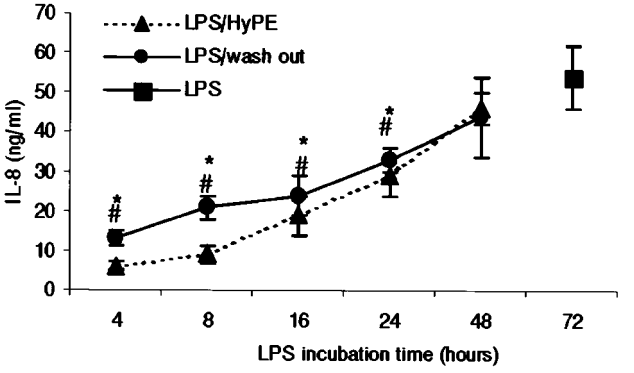

Figure 8 Function of LPS mediated chemokine production by HyPE. LMVEC were either stimulated with LPS (1 μg ml^{-1} well) (boxes) or at the indicated time after LPS stimulation HyPE was added to the wells (triangles) or cell culture supernatants were collected (LPS was washed out) and normal medium was added (points). The culture medium in all wells was collected after 72 h and assessed for IL-8 production by ELISA. Each value represents the mean production of triplicate cultures ± s.d. *$P<0.05$ LMVEC pretreated with LPS before HyPE addition compared to cells that were stimulated only with LPS. #$P<0.05$ LMVEC pretreated with LPS before washing compared to cells that were stimulated only with LPS.

production by LMVEC induced by TNF-α or IL-1, which are also strong stimuli of endothelial chemokine production during inflammatory processes like ARDS (data not shown). It thus seems that steric hindrance by the ExPLIs can explain in part the ExPLIs inhibitory effect on LPS-induced chemokine production.

Discussion

sPLA$_2$ has been assumed to play an important role in the development of ARDS, as elevated PLA$_2$ activity in the serum of patients with septic shock was found to be associated with an increased risk for the development of

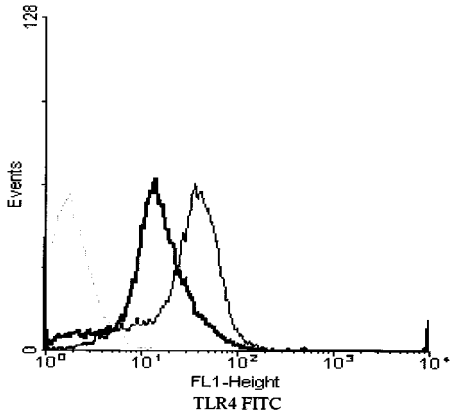

Figure 9 Effect of HyPE on binding of anti TLR4 antibodies. LMVEC were harvested as described and preincubated (fat line) or not (thin line) for 20 min with HyPE (10 μM). Thereafter monoclonal antibodies against TLR4 were added to the cell suspension and FACS-analysis was performed as described in Methods. Grey line = negative control.

ARDS (Vadas & Pruzanski, 1993), and increased $sPLA_2$ levels in bronchoalveolar-fluid correlated with the severity of ARDS (Offenstadt *et al*., 1981). This was supported by the finding that $sPLA_2$ infusions into the pulmonary circuit of dogs and rats (Littner & Lott, 1990) induce changes resembling those observed in ARDS, and intratracheal administration of $sPLA_2$ caused similar histological injury and clinical symptoms (Edelson *et al*., 1991).

In accordance with this, studies with different cultured cells have suggested that the cellular stimulation by LPS *per se* involves the activation of $sPLA_2$. It has thus been assumed that $sPLA_2$ acts as an important mediator of pathophysiology of endotoxin-induced ARDS (Partrick *et al*., 2001). Accordingly, inhibition of $sPLA_2$ activity has been considered for the treatment of ARDS. A number of inhibitors have been proposed and tested *in vitro* for this purpose, but were found unsatisfactory or toxic when tested *in vivo* (Yokota *et al*., 1999; Song *et al*., 1999).

However, the present study suggest that in LMVEC stimulation by endotoxins does not involve $sPLA_2$, since no activation or secretion of PLA_2 were observed upon treatment of these cells with LPS or by highly inflammatory cytokines combinations. This was supported by the findings that the specific $sPLA_2$ inhibitor LY311727 had no effect on the production of chemokines or adhesion molecules in LMVEC. It should be stressed that PLA_2 activation in endothelial cells can be observed when appropriate stimuli are used. However, data on endothelial $sPLA_2$-activity are inconclusive (Wong *et al*., 2000) and the formation of eicosanoids in endothelial cells may be mainly attributed to the activity of $cPLA_2$ (Doerfler *et al*., 1994; Fouda *et al*., 1995). This does not rule out the possibility that exogenous $sPLA_2$, secreted by other cells or tissues in body fluid of ARDS patients, may affect LMVEC by inducing the production of inflammatory mediators by these cells.

The present study raises particular interest in the ExPLIs, which are composed of a PLA_2 inhibiting phospholipid that incorporates into the cell membrane (Dan *et al*., 1998), linked to a GAG-mimicking polymer, which prevents its internalization. This structure makes them potent inhibitors of endogenous PLA2 activation, by LPS (Balsinde *et al*., 2000) and other inflammatory agents (Yedgar *et al*., 1995; Ginsburg *et al*., 1997), and protectors of cells from exogenous inflammatory mediators, such as ROS, hemolysins and $sPLA_2$ (Ginsburg *et al*., 1997; Dan *et al*., 1998; Schnitzer *et al*., 2000; Yard *et al*., 2002). In the present study it was found that the ExPLIs dramatically suppressed LTA and LPS-induced production of CXC chemokines and expression of adhesion molecules. Since, as shown in this study, the action of endotoxins on LMVEC is independent of PLA_2 activity, it follows that the inhibition of the LPS responses by the ExPLIs is not due to their PLA_2-inhibiting capacity, but *via* a different mechanism. Since the TNF-α- induced NF-κB activation was not influenced by HyPE, it seems that the ExPLIs interfere with the LPS signalling pathway.

Figure 8 shows that LPS-induced chemokine production in LMVEC is proportional to stimulation duration; the stimulation can be stopped by washing LPS away or by addition of HyPE, thus suggesting that HyPE competes with LPS. This was supported by the finding that the binding of TLR to its antibody is reduced by HyPE (although not completely). This of course does not necessarily rule out binding of LPS with its receptor, but if we interpolate from the TLR antibody to LPS, it may be suggested that the ExPLIs interfere with the interaction of endotoxins with the receptor at the cell surface. However, the LPS action was inhibited as well by different ExPLIs, in which the inhibiting lipid is linked to polymeric carriers that differ in size and chemical nature or to aspirin (Figure 10). This suggests that simple steric hindrance does not fully explain the mechanism by which the ExPLIs suppress endotoxin-induced chemokine production by LMVEC.

LPS is an amphipathic, negatively-charged molecule, and it has long been proposed that its action involves ionic interaction with the membrane and intercalation of its lipid moiety (lipid A) into the lipid bilayer, in addition to receptor binding (Jacobs *et al*., 1990). The ExPLIs used in the present study (including ASPE) are negatively charged, and anchored to the cell membrane *via* their lipid moiety (PE). It is thus not unlikely that the ExPLIs interfere as well with these LPS interactions with the membrane and/or with the incorporation of Lipid A into the lipid bilayer, but the exact mechanism of the ExPLIs' inhibitory effect has yet to be explored.

The present study demonstrates that the inhibitory effect of the ExPLIs is expressed at mRNA level of the chemokines and adhesion molecules. Moreover, this inhibitor suppresses the activation of NF-κB, a transcription factor that mediates LPS-induced gene expression.

In summary, the ExPLIs are potent protectors of cells from damage by exogenous $sPLA_2$s and other cell inflammatory agents (Yedgar *et al*., 1995; Ginzburg *et al*., 1997; Dan *et al*., 1998; Schnitzer *et al*., 2000; Balsinde *et al*., 2000; Yard *et al*., 2001). Although the exact mechanism of the ExPLIs action in LMVEC remains to be investigated, all-in-all, these findings present the ExPLIs as potent inhibitors of endotoxin-induced cellular responses, and may introduce a novel approach in the research and therapy of ARDS.

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