Lysophosphatidylcholine impairs endothelial barrier function through the G protein-coupled receptor GPR4

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LPC mediates these activities remain unclear and controversial. Recent evidence implicates involvement of a novel subfamily of G protein-coupled receptors (GPR4, G2A, OGR1, and TDAG8) that are sensitive to lysolipids and protons. We previously reported that one of these receptors, GPR4, is selectively expressed by a variety of endothelial cells and therefore hypothesize that the LPC-stimulated endothelial barrier dysfunction is mediated through GPR4. We developed a peptide Ab against GPR4 that detected GPR4 expression in transfected COS 7 cells and endogenous GPR4 expression in endothelial cells by Western blot. Endothelial cells infected with a retrovirus containing small interference RNA (siRNA) to GPR4 resulted in 40–50% decreased GPR4 expression, which corresponded with partial prevention of the LPC-induced J decrease in transendothelial resistance, 2) stress fiber formation, and 3) activation of RhoA. Furthermore, coexpression of the siRNA-GPR4 with a siRNA-resistant mutant GPR4 fully restored the LPC-induced resistance decrease. However, extracellular pH of <7.4 did not alter baseline or LPC-stimulated resistances. The results provide strong evidence that the LPC-mediated endothelial barrier dysfunction is regulated by endogenous GPR4 in endothelial cells and suggest that GPR4 may play a critical role in the inflammatory responses activated by LPC.

endothelial resistance; small interference ribonucleic acid; actin; RhoA

OVER THE PAST 15–20 YEARS, abundant evidence has accumulated documenting direct proinflammatory and atherogenic effects of lysophosphatidylcholine (LPC). In the vascular endothelium, LPC upregulates a range of proinflammatory molecules, such as adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule, and P-selectin; see Refs. 17, 26, 44), which are accompanied by increased leukocyte-endothelial adhesion (17, 26). LPC also activates increased production and release of cytokines (28, 37), superoxide anion (16), and matrix metalloproteinase-2 (12).

LPC is mostly derived from membrane phosphatidylcholine by two sources. The circulating LPC is generated predominantly from the activity of lecithin-cholesterol acyltransferase, which transfers a fatty acid from phosphatidylcholine to cholesterol (36). Also, phospholipase A2 (PLA2) hydrolyzes phosphatidylcholine, simultaneously generating a molecule of LPC and an arachidonic acid (23, 39). The high content of LPC within oxidized low-density lipoprotein particles is believed to be attributed to the association of type VIIA (Ca2+-independent) PLA2 (6). Elevated LPC levels have been reported in several diseases, such as endometriosis (27), ovarian cancer (30), asthma (24), rhinitis (24), the ischemic myocardium, and atherosclerotic aortas (13, 34). Despite this abundant evidence for the proinflammatory nature of LPC, the mechanisms by which this occurs are controversial, and, in the vascular endothelium, the mechanisms are even less clear. The current hypothesis is that LPC may act by way of a novel subset of G protein-coupled receptors [i.e., GPR4, G2A (G2 accumulation), OGR1 (ovarian cancer G protein-coupled receptor 1), and TDAG8 (T cell death-associated gene 8)] that are sensitive to lysolipids and protons (19, 38, 41). Of these members, GPR4 gene expression is reported to have the widest tissue distribution, showing high expression in ovary, liver, lung, kidney, lymph node, placenta, skeletal muscle, and subthalamus nucleus (41). The other members have a more limited tissue distribution, with OGR1 found in the placenta, lung, spleen, and testis, whereas G2A and TDAG8 are limited to lymphoid tissues (41). Our own work shows that endothelial cells from different vascular beds selectively expressed GPR4 over either G2A, OGR1, or TDAG8 (14, 21).

We report that direct stimulation of endothelial cells with LPC causes increases in endothelial permeability that are dependent, in part, on RhoA and protein kinase C-dependent, in part, on RhoA and protein kinase C-signal cross talks (11). Furthermore, we found that increased surface binding of [3H]LPC corresponded to increased levels of GPR4 mRNA (21), suggesting that the LPC-induced increased permeability may be attributed to regulation through GPR4. These findings appear consistent with reports in which GPR4-transfected cells show binding for LPC with a dissociation constant of 159 nM, LPC-induced activation of serum response element extracellular signal-regulated kinase (ERK), and receptor internalization (43). Other reports, in contrast, found that LPC did not stimulate the binding of GTPγS to membranes prepared from GPR4-transfected cells nor activated ERK (4) and that lowering pH to 7.2 resulted in increased intracellular cAMP levels (19). Therefore, it remains unclear whether LPC-mediated inflammatory activities of the vascular endothelium are regulated through endogenous GPR4 expression.

We investigated the specific hypothesis that the LPC-stimulated endothelial barrier dysfunction is mediated through GPR4. For study, we developed a peptide antibody (Ab) against GPR4, which detected GPR4 expression in endothelial...
cells. The small interference RNA (siRNA)-mediated silencing of GPR4 expression corresponded with partial prevention of the LPC-induced decrease in transendothelial resistance, and coexpression of the siRNA-GPR4 with an siRNA-resistant mutant GPR4 (mtGPR4) fully restored the LPC-induced resistance decrease. Furthermore, the knock down of GPR4 prevented both stress fiber formation and activation of RhoA in response to LPC stimulation. The results provide strong evidence that the LPMC-mediated endothelial barrier dysfunction is regulated by endogenous GPR4 in endothelial cells.

MATERIALS AND METHODS

Materials. The following reagents were purchased as follows: from Amersham Pharmacia Biotech (Piscataway, NJ): ECL kit, glutathione Sepharose 4B beads, and horseradish peroxidase-conjugated (HRP) anti-rabbit IgG antibodies; from Invitrogen (Carlsbad, CA): polyclonal anti-RhoA Ab and monoclonal anti-actin Ab; from BD Biosciences (Palo Alto, CA): polyclonal anti-green fluorescent protein (GFP) Ab; from Sigma Chemical (St. Louis, MO): human β-casein gene and resuspended in sufficient volume of HBSS to give a final concentration of 1 mM. The samples were vortexed at room temperature for 1 min (2 times) to yield a clear dispersion, and the final concentration was confirmed by analysis of lipid phosphorus by the modified Bartlett procedure (22). The preparations were stored at 4°C and were used within 60 days.

Ab production. A polyclonal Ab against GPR4 was made with synthesis of a peptide corresponding to the COOH terminus of human GPR4 (GenBank no. U21051) by solid phase peptide synthesis with 9-fluorenylmethoxycarbonyl chemistry (Research Resources Core Facility, University of Illinois, Chicago, IL). The peptide was verified by HPLC chromatography and NH2-terminal sequencing. After conjugation to keyhole limpet hemocyanin, the peptide was injected in rabbits for immunization. Blood was collected before injection to obtain preimmune serum, booster injections were given at 4-wk intervals, and blood was collected 3–4 wk after each immunization.

The immunogenicity of the harvested antiserum was confirmed by indirect ELISA using microtiter plates precoated with GPR4 COOH-terminal peptide. Serial dilutions of the harvested anti-GPR4 peptide antiserum or dilutions of preimmune serum were added to appropriate wells, followed by incubation of goat anti-rabbit IgG conjugated to alkaline (7, 21, 35). Bovine pulmonary microvessel (BPMEC) and pulmonary artery (BPAEC) endothelial cells were purchased from VecTechologies (Rensselaer, NY) and serially cultured up to passage 12 and 22, respectively, for studies, as previously described (11, 20). The culture medium for BPMEC contained DMEM supplemented with 20% FBS (15 μg/ml) and 1% nonessential amino acids; that for BPAEC contained MCDB 131 medium supplemented with 10% FBS, 10 ng/ml EGF, 0.1 mg/ml heparin, 1 μg/ml hydrocortisone, 1% penicillin-streptomycin, and 1% l-glutamine. The COS 7 cells and 293T cells were maintained in DMEM containing 5% FBS and 1% penicillin-streptomycin.

Preparation of LPC. LPC (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids (Alabaster, AL) and prepared as previously described (11, 21). Stock LPC was dissolved in chloroform-methanol (2:1) and stored at −20°C. The LPC was checked for fatty acid composition by gas liquid chromatography and found to be at least 96% pure. Aliquots of these were prepared for experimental use by evaporation to dryness under nitrogen gas and resuspended in sufficient volume of HBSS to give a final concentration of 1 mM.

The immunogenicity of the harvested antiserum was confirmed by indirect ELISA using microtiter plates precoated with GPR4 COOH-terminal peptide. Serial dilutions of the harvested anti-GPR4 peptide antiserum or dilutions of preimmune serum were added to appropriate wells, followed by incubation of goat anti-rabbit IgG conjugated to alkaline

Fig. 1. Affinity-purified anti-G protein-coupled receptor 4 (GPR4) antibody (Ab) detects GPR4-green fluorescent protein (GFP) fusion protein. COS 7 cells were transfected with the plasmid, pEGFP-N1–3HA–GPR4, to overexpress GPR4-GFP fusion protein. Affinity-purified anti-GPR4 Ab (diluted at 1:200) was used for Western blot analysis of the COS 7 cell lysate. Representative Western blot shows detection of GPR4-GFP fusion protein at the predicted molecular mass (top); the reprogred membrane with anti-GFP Ab (bottom) shows GFP in transfected cells only. C, control nontransfected COS 7 cells; Mock, transfection in the absence of the plasmid pEGFP-N1–3HA–GPR4; n = 3 experiments.

Fig. 2. Endothelial cells express endogenous GPR4. A: Western blot analysis using affinity-purified anti-GPR4 Ab (diluted at 1:100) detected a band at −45 kDa of cell lysates from human brain microvascular endothelial cells (HBMEC) and human dermal microvascular endothelial cells (HMEC). B: top: Western blot showing anti-GPR4 Ab detection of GPR4 at different loaded amounts of HMEC lysates (7.5 and 15 μg). Bottom: negative control in which the anti-GPR4 Ab was precomplexed with GPR4. COOH-terminal peptide before Western blot analysis; n = 2 experiments.
phosphatase for reaction with the substrate p-nitrophenyl phosphate. The reaction was detected by reading absorbency at 405 nm for immunogenicity evaluation. For studies, the anti-peptide serum was purified by routine peptide affinity column chromatography (anti-GPR4 Ab). In brief, the peptide was coupled to Sepharose 4B gel in ligand coupling buffer (0.1 M NaHCO₃, pH 8.3, containing 0.5 M NaCl) and loaded in a 10-cm column; the Ab was eluted with glycine buffer, pH 2.5 (50 mM glycine·HCl, pH 2.5, 0.1% Triton X-100, and 0.15 M NaCl) and desalted in PD-10 columns.

Production of retrovirus. Retrovirus plasmids containing siRNA targeted to GPR4 or LPA₃ and mtGPR4 were generously provided by Dr. Yan Xu (The Cleveland Clinic Foundation, Cleveland, OH). The retrovirus plasmids were constructed as described (14). In brief, the pGEM1 plasmid containing the U6 promoter was used as the template for PCR reactions [primers 5'-AGATCTGATTTAGGTGACACTATAG-3' and 5'-AAAAAAAGGACAAATTCAGGCCCAGCGTAAAGGAAACCGCAAGCTTCGGTCCCCACACTGGGCTGGAATTGCCTCGGTGTTTCGTTCTTTCCAAA-3' (for siRNA-LPA₃) or 5'-AAAAAAAGTGCGACAGCACCCTCTCAACTACACCCCAAGCTTTGGTGTCAGCTAAGATGCTGCCCAGCAGCTTCTGTCTTTCCAAA-3' (for siRNA-GPR4)]. The PCR products were then subcloned into the pMSCVpuro vector (Clontech Laboratories, Palo Alto, CA), generating, respectively, pMSCVpuro-siRNA-LPA₃ and pMSCVpuro-siRNA-GPR4. The retrovirus containing the mtGPR4 was made with primers 5'-CGCAGATCTATGGGCAACCACGTGG (the 5'-sense primer) and 5'-TCATTGAGCAGGAGGGAGCATTTTGAGTTGGACCTGGTCC (the 3'-antisense primer), which were used to amplify the complete coding region of the GPR4 gene for subcloning into the pMSCVpuro vector (pMSCVpuro-mtGPR4). The mtGPR4 transcripts were constructed by changing eight nucleotides in the region of GPR4 targeted by siRNA molecule without changing the encoded amino acids, resulting in expression of full-length wild-type GPR4, which was resistant to the siRNA-targeted degradation.

To produce recombinant retroviruses (siRNA-GPR4, siRNA-LPA₃, and mtGPR4) for infection of endothelial cells for study, 293T cells were grown to 70–80% confluence in T75 flask for cotransfection of 12 µg either pMSCVpuro-siRNA-GPR4, pMSCVpuro-siRNA-LPA₃, or pMSCVpuro-mtGPR4 with 12 µg pAmpho (amphotropic retrovirus packaging plasmid; Clontech Laboratories) using Lipofectamine reagent. After 24 h, the medium was replaced with...
activation, as previously described (11, 33). In brief, glutathione-
RhoA was determined by affinity binding assay to evaluate RhoA
expression on sterile, fibronectin-coated 10 electrode/well arrays, and resistance was measured as previously described (11, 33). Initial baseline resistance typically showed >800 Ω, which is equivalent to a calculated 4.0 Ω/cm², after correction for the 10 electrode/well configuration (Applied BioPhysics). Cell monolayers with <800 Ω were rejected from study. The endothelial cells were challenged with reagents according to experimental protocol, and resistance was recorded continuously for up to 4 h.

Affinity binding assay for RhoA-GTP. The GTP-bound form of RhoA was determined by affinity binding assay to evaluate RhoA activation, as previously described (11, 33). In brief, glutathione-S-transferase (GST)-C21 fusion protein (rhotekin, a Rho target mole-
cule) was prepared from induction of cultures of transformed Esche-
richia coli with 0.1 mM isopropylthiogalactoside. Endothelial cells were grown in six-well dishes to confluence, treated according to the experimental protocol, and collected in GST-FISH buffer [50 mM Tris (pH 7.4), 10% glycerol, 100 mM NaCl, 1% Nonidet P-40, 2 mM MgCl₂, 25 mM NaF, and 1 mM EDTA] plus protease inhibitor cocktail (10 μg/ml pepstatin A, 10 μg/ml each of aprotinin and leupeptin, and 1 mM PMSF). Cell lysates were pelleted by centrifugation at 10,000 g at 4°C for 5 min, and equal volumes of supernatant were incubated with purified GST-rhotekin coupled to glutathione Sepharose 4B beads at 4°C for 1 h. The GTP form of RhoA bound specifically to the rhotekin-Sepharose beads was eluted by boiling in 2.5X Laemmli sample buffer and electrophoresed on 12.5% SDS-
PAGE, and Western blots were made with anti-GFP purified Ab di-
rected against RhoA.

Fluorescence microscopy. Endothelial cells were plated on cover slips for routine immunofluorescence preparation. For actin visualization, endothelial cells were fixed in 3% paraformaldehyde in PBS plus 2% sucrose for 15 min at room temperature. The cells were perme-
bilized with HEPES-Triton X-100 buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, and 0.5% Triton X-100) for 5 min at 4°C, followed by phalloidin conjugated with rhodamine red. The slides were sealed with Aquamount medium (Lerner Laborato-
ries, Pittsburgh, PA). For routine preparation of immunofluorescent
localization of GPR4 in brain tissue, cryosections (10–15 μm thick) of brain biopsies from epileptic patients were made. In brief, the sections were incubated with primary anti-GPR4 Ab (1:200) and goat anti-GLUT1 Ab (1:50) at 4°C overnight for colocalization studies. The sections were next incubated with secondary anti-rabbit IgG Ab conjugated with Texas red (for detection of GPR4) and anti-goat IgG Ab conjugated with FITC (for detection of GLUT1). Slides were examined using the Olympus Confocal Fluoview Microscope equipped with krypton (Olympus America, Melville, NY).

Western blots. Endothelial cells were grown to confluence and treated according to the experimental protocol. The cells were washed two times with PBS and collected in the appropriate extraction buffer, and protein concentration was determined using the BCA Protein Assay kit with BSA as standard. The cell lysates were loaded at constant protein concentrations, separated by SDS-PAGE in 10–12% acrylamide gels, and electrotransferred to nitrocellulose membranes. The membrane was blocked with 5% nonfat dry milk in TBS with 0.05% Tween 20 (TBST) and then incubated with appropriate primary antibodies diluted in TBST with 1% nonfat dry milk overnight at 4°C in a rocker. The blot was washed five times with TBST and incubated with the appropriate anti-IgG secondary Ab conjugated with horseradish peroxidase. The bands were detected using the ECL kit.

Statistics. Single-sample data were analyzed by the two-tail t-test; a multiple-range test (Scheffé’s test) was used for comparison of experimental groups with a single control group.

RESULTS

Endothelial cells express endogenous GPR4. The specificity of the anti-GPR4 Ab in detection of GPR4 in cells was evaluated by multiple approaches. COS 7 cells were transfected with the plasmid pEGFP-N1–3HA-GPR4 to overexpress GPR4. Western blot analysis of the COS 7 cell lysate indicated that anti-GPR4 Ab detected high expression only in cells transfected with pEGFP-N1–3HA-GPR4, but not in mock transfectants or nontransfected cells (Fig. 1). Bands of slightly higher molecular mass were detected from all groups, which were likely nonspecific. The membrane was stripped and reprobed with anti-GFP Ab, which showed that only the transfected cells expressed GFP protein, consistent with detection of GPR4 expression in the transfectants (Fig. 1).

From endothelial cells, Western blot analysis with the anti-GPR4 Ab detected a strong band at ~45 kDa, the
predicted molecular mass of GPR4 (Fig. 2A). Increasing the amount of protein loading for the SDS-PAGE corresponded with increased intensity of the band (Fig. 2B, top), whereas precomplexing the anti-GPR4 Ab with the GPR4 COOH-terminal peptide antigen before the Western blot procedure resulted in absence of the band (Fig. 2B, bottom). The results indicated specificity of the anti-GPR4 Ab in detection of endogenous GPR4 in cells. In situ immunofluorescence localization studies showed that GPR4 (red fluorescence) distributed primarily in blood vessels in human brain tissue sections (Fig. 3). The corresponding overlay with immunofluorescence of GLUT1 transporter (green fluorescence), used as an indicator of a transmembrane protein, showed overlap of signals (yellow fluorescence), indicating colocalization of the two proteins in the cerebral blood vessels (Fig. 3). 4’,6-Diamidino-2-phenyindole staining (blue fluorescence) indicated general distribution of cell nuclei in the brain sections (Fig. 3).
In the next studies, siRNA was used to posttranscriptionally silence the endogenous endothelial GPR4 expression. Endothelial cells were infected with the retrovirus containing siRNA-GPR4 overnight, and the cells were collected for Western blot analysis with anti-GPR4 Ab. The retrovirus, siRNA-LPA3 targeted to LPA3 (a GPCR for the specific ligand LPA), was used as a negative control. Results indicated that siRNA-GPR4 infection of HMEC decreased 40–50% GPR4 expression (Fig. 4). The Western blot membrane was reprobed with anti-β-actin Ab, which showed similar bands from the experimental groups, indicating equal loading of the protein lysate. With the control groups, HMEC infected with siRNA-LPA3 expressed similar levels of GPR4 as noninfected control (Fig. 4). Overall, the results indicated that the siRNA-GPR4-mediated decrease in GPR4 expression was not attributed to retrovirus infection per se, but was specific for GPR4.

GPR4 dependency of the barrier dysfunction response. Direct stimulation of HMEC, HBMEC, BPMEC, and BPAEC with 1–10 μM LPC caused rapid reversible decreases in the transendothelial electrical resistance (Fig. 5), which confirmed our previous reported observations (11). However, the pattern and sensitivity of the resistance decrease was different among the four endothelial cell types (Fig. 5). The effects of GPR4 knock down on the LPC-induced resistance decrease were determined by infection overnight with siRNA-GPR4 of HMEC grown to confluence in the resistance electrodes and then resistance change in response to LPC stimulation was made. The results showed that siRNA-mediated knock down of GPR4 corresponded to ~50% inhibition of the LPC-induced resistance decrease (Fig. 6). The retrovirus infection alone had negligible effects on the basal resistance, since both noninfected and siRNA-GPR4-infected groups showed similar baselines before LPC challenge. In the control group in which the endothelial cells were infected with siRNA-LPA3, the LPC-induced resistance decrease was similar to noninfected cells (Fig. 6). To test whether siRNA-LPA3 was functional, the infected cells were stimulated with LPA (30 μM), and effects on resistance were determined. Results showed that LPA stimulation increased resistance above baseline in HMEC; however, in cells infected with siRNA-LPA3, the resistance increase was inhibited significantly (Fig. 7).

We further tested the requirement of GPR4 for the LPC-induced endothelial barrier dysfunction response by use of the

![Fig. 6. LPC-decreased endothelial resistance is dependent on GPR4. Confluent monolayers of HMEC grown on resistance electrodes were infected overnight with the retrovirus siRNA-GPR4 or siRNA-LPA3 and stimulated with 2 μM LPC (arrow), and the resistance was recorded for up to 2–3 h. A: representative graph showing resistance (Ω) change in real time. B: summary graph from 15–17 separate determinations showing the maximal resistance decrease from baseline. *P < 0.01 compared with noninfected control (Control).]
mtGPR4 to restore the siRNA-mediated inhibition of resistance decrease. The mtGPR4 encodes the wild-type GPR4 but is resistant to the siRNA-targeted degradation (see MATERIALS AND METHODS). In these studies, endothelial cells were coinfected with the siRNA-GPR4 and mtGPR4 retroviruses and subsequently stimulated with LPC, and transendothelial resistance was measured as described. With coexpression of mtGPR4, the LPC-induced resistance decrease was similar to that of control cells (Fig. 8), indicating that the mtGPR4 effectively prevented the siRNA-mediated effect and restored the LPC-induced resistance decrease. Infection with the mtGPR4 virus alone showed a slight and nonsignificant increase in response to LPC compared with noninfection control.

We also investigated whether lowering pH below 7.4 would affect endothelial barrier function. Endothelial cells grown to confluence on the resistance electrodes were incubated in medium with pH adjusted to 6.5, 7.0, 7.5, or 8.0, and basal resistance was monitored for >3 h. During this period, baseline resistance of the four groups remained stable and similar (Fig. 9). After this period, the cells were stimulated with 2 μM LPC, and resistance was further monitored for an additional 2–3 h. Results showed that, within the pH 6.5–8.0 range, the stimulated resistance decrease was similar for all groups (Fig. 9).

LPC induces GPR4-dependent actin remodeling mechanisms. We previously observed that the LPC-induced increase in endothelial permeability is regulated in part by RhoA (11), suggesting an actin-myosin mechanism of barrier dysfunction. To investigate this possibility, we determined the effects of LPC on actin stress fiber formation in endothelial cells. In HMEC, direct stimulation with 2 μM LPC resulted in increased stress fibers within 10 min (Fig. 10, top), which were sustained for up to 60 min (Fig. 10, middle). However, by 2 h, the stress fibers were decreased toward control levels (Fig. 10, middle). These results are consistent with our previous finding that LPC activates RhoA (11) and suggest that RhoA regulated the LPC-mediated actin remodeling in the current study. To test whether the LPC-induced increased stress fiber formation was dependent on GPR4, HMEC were infected with the retrovirus siRNA-GPR4 as described for the resistance studies and then stimulated with LPC for 10 min. The results showed that siRNA-GPR4 prevented the formation of the LPC-induced actin stress fibers (Fig. 10, bottom). Infection with siRNA-GPR4 per se did not alter the actin stress fiber organization. Furthermore, the effects of LPC on RhoA activation in endothelial cells were determined by affinity binding assay. As we expected, LPC stimulation caused RhoA activation in endothelial cells (Fig. 11A), confirming our previous observations. However, endothelial cells infected with siRNA-GPR4 inhibited the LPC-stimulated RhoA activation (Fig. 11B).
Fig. 8. Mutant GPR4 (mtGPR4) restores LPC-induced resistance response. Confluent monolayers of HMEC grown on resistance electrodes were infected overnight with siRNA-GPR4 or mtGPR4 or coinfected with siRNA-GPR4 plus mtGPR4. Cells were stimulated with 2 μM LPC (arrow), and the resistance was recorded for up to 2–3 h. A: representative graph showing resistance (Ω) changes in real time. B: summary graph of maximal resistance decrease from baseline analyzed from 15–17 separate determinations. *P < 0.01 compared with noninfected control (Control).

Fig. 9. Effects of extracellular pH on resistance. Confluent monolayers of HMEC grown on resistance electrodes were incubated with medium containing different pH conditions for >3 h (double arrows) and then challenged with 2 μM LPC (arrow), and the resistance was recorded for up to 2–3 h. Shown is a representative graph from 3 separate determinations.
DISCUSSION

The findings from this study provide critical evidence for the regulation of the LPC-induced endothelial barrier dysfunction by GPR4. We showed that siRNA-mediated knock down of endogenous GPR4 corresponded with prevention of ~50% of the resistance decrease in response to LPC stimulation. This partial inhibition may be attributable to GPR4-independent mechanisms, since some LPC-mediated activities have been reported to be sensitive to platelet-activating factor receptor antagonism (29). However, we found that the ability of the retrovirus siRNA-GPR4 to knock down GPR4 expression was only 40–50% compared with controls, which would be consistent with the partial inhibition of the LPC-induced barrier dysfunction observed. Importantly, the siRNA-mediated inhibition of the barrier response was fully prevented by rescue with the mtGPR4 that encodes functional GPR4. This latter finding provides strong evidence that the LPC-induced barrier dysfunction was specifically dependent, at least in part, on endogenous GPR4. Overall, the current results are consistent with our previous report that increased GPR4 gene expression in endothelial cells corresponded with increased specific surface binding of [3H]LPC (21).

We report that direct stimulation of different endothelial cell types (HMEC, HBMEC, BPAEC, and BPMEC) with LPC rapidly impaired the barrier function, which recovered toward baseline within ~30 min, although the pattern of the response was different among the cell types. The finding extends our previous similar report to include effects of LPC on other endothelial cell types and suggests that LPC is a ubiquitous inflammatory mediator of the vascular endothelium. The mechanisms by which LPC impairs the endothelial barrier were found to be associated with activation of RhoA and formation of actin stress fibers, all supportive of an endothelial contractile mechanism (5, 15, 31). We (11) and others (42) have reported that LPC activates RhoA in endothelial cells, and inhibition of RhoA prevented at least, in part, the LPC-induced barrier dysfunction (11). The contractile mechanism is believed to be a major determinant underlying barrier dysfunction induced by several inflammatory mediators, including thrombin (5, 31).

We observed that siRNA-mediated knock down of GPR4 inhibited the LPC-induced activation of RhoA, supporting the possibility that the GPR4-dependent regulation of barrier function is through RhoA signaling. Although we (11) and others (42) have reported that LPC activates RhoA, the current finding provides the first evidence for an endogenous receptor-mediated regulation. At present, the G proteins coupled to the endogenous GPR4 in endothelial cells are not known. Studies based on ectopic expression of GPR4 suggest that the receptor is likely coupled to multiple G proteins, particularly Gq and Gs; however, which G proteins are responsible for the transduction of Rho signaling remains to be determined. The finding that inhibition of the LPC-induced actin stress fiber formation occurred also in siRNA-GPR4 cells is consistent with the corresponding inhibition of RhoA activation. RhoA is a known potent regulator of stress fiber formation in cells (10, 45),

Fig. 10. LPC-induced stress fiber formation is dependent on GPR4. HMEC grown to confluence on glass coverslips were stimulated with 2 μM LPC for different periods of time (10, 60, and 120 min) and prepared for fluorescence detection with phalloidin conjugated with rhodamine red, as described in MATERIALS AND METHODS. In a separate group, HMEC were infected overnight with siRNA-GPR4 and then stimulated with LPC for 10 min. Original magnification ×40. Scale bar is 15 μm; n = 3.

Fig. 11. Regulation of LPC-induced RhoA activation by GPR4. RhoA activation in endothelial cells was determined by affinity binding assay (MATERIALS AND METHODS). A: confluent monolayers of HMEC and HBMEC were stimulated with LPC (5 μM) for 10 min, and RhoA-GTP was determined; n = 3. B: HBMEC were infected with siRNA-GPR4 overnight as described and then stimulated with 5 μM LPC for 10 min; n = 3.
regulating several fundamentally important activities, including endothelial permeability (5, 8), angiogenesis (18), apoptosis (32), and cell migration (3).

It is possible that the LPC-induced endothelial barrier dysfunction is attributed to an indirect effect of LPA, since LPC can be metabolized by extracellular lysophospholipases (i.e., lysophospholipase D) to other bioactive lysolipids such as LPA (40). However, our present observations argue against such possibility. First, in contrast to LPC, LPA stimulated an increase in resistance in HMEC, a finding consistent with reports by others for bovine aortic endothelial cells (2), BPAEC (9), (25), and BPMEC (25). Second, endothelial cells infected with siRNA-LPA3 responded to LPC stimulation with a resistance decrease comparable to control noninfected cells. Third, the LPA-induced resistance increase was inhibited in the siRNA-decrease comparable to control noninfected cells. Third, the LPA-induced endothelial barrier dysfunction was a distinct response independent of the LPA3 receptor.

GPR4 belong to a novel subfamily of GPCRs believed to be responsive to both lysolipids and protons (19, 38). Therefore, we investigated whether the GPR4-dependent endothelial barrier function was also regulated by extracellular proton concentration. We found that pH conditions of medium between 6.5 to 8.0 did not alter baseline resistance or the LPC-stimulated resistance decrease, suggesting that endothelial barrier function was insensitive to changes in the extracellular proton concentrations. The current observation is consistent with our previous report that pH of <7.4 does not regulate intracellular cAMP levels in parental or GPR4-transfected endothelial cells (14).

In summary, we developed a peptide Ab against GPR4, which detected endogenous GPR4 expression in endothelial cells. The siRNA-mediated silencing of GPR4 expression corresponded with partial prevention of the LPC-induced decrease in transendothelial resistance, and coexpression of siRNA-GPR4 with a siRNA-resistant mtGPR4 fully restored the LPC-induced resistance decrease. Furthermore, the knock down of GPR4 prevented both stress fiber formation and activation of RhoA in response to LPC stimulation. The results provide strong evidence to suggest the LPA-mediated endothelial barrier dysfunction was regulated by endogenous GPR4 in endothelial cells and suggest that GPR4 may play a critical role in the inflammatory responses activated by LPC.

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