Lysophospholipids Enhance Matrix Metalloproteinase-2 Expression in Human Endothelial Cells

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Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (SIP) are both low-molecular-weight lysophospholipids, which promote cell proliferation, migration, and invasion via interaction with a family of specific G protein-coupled receptors. Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes, which are involved in degradation of the extracellular matrix and play critical roles in endothelial cell migration and matrix remodeling during angiogenesis. Among these MMPs, MMP-2 is known to trigger cell migration. In the present study, we examined the effects of LPA and SIP on MMP-2 expression in human endothelial cells. We showed that LPA and SIP enhanced MMP-2 expression in mRNA, protein levels, and also enzymatic activity of cells of the EAhy926 human endothelial cell line. The enhancement effects occurred in concentration- and time-dependent manners. Results from real-time PCR, Western blots, and substrate gels indicated that these enhancement effects were mediated through MAPK kinase/ERK-, nuclear factor-κB-, and calcium influx-dependent pathways. Furthermore, we show that endothelial cell invasion of the gel was enhanced by lysophospholipids, and the induction could be prevented by an MMP inhibitor, GM6001. These observations suggest that LPA and SIP may play important roles in endothelial cell invasion by regulating the expression of MMP-2. (Endocrinology 146: 3387–3400, 2005)

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Abbreviations: ECM, Extracellular matrix; Edg, endothelial differentiation gene; FBS, fetal bovine serum; GAPDH, glyceraldehyde phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; LPA, lysophosphatidic acid; LPL, lysophospholipid; M199, medium 199; MEK, MAPK kinase; NF-κB, nuclear factor-κB; MMP, matrix metalloproteinase; PDTC, pyrrolidinedithiocarbamate; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PI2, 4-amino-5-(4-chlorophenyl)-7-(4-butyl)pyrazolo(3, 4-d) pyrimidine; SIP, sphingosine 1-phosphate; TIMP, tissue inhibitor of metalloproteinase; uPA, urokinase-type plasminogen activator.

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The major sources of LPA and SIP are activated platelets, injured cells, and cells stimulated by growth factors, suggesting potential roles in inflammation, wound healing, and tumor formation (6). The concentration of SIP is estimated to be 200 nM in plasma serum (20), and that of LPA is approximately 1–10 μM in human serum with variable reports of plasma concentrations from undetectable to 2 nM (21). The growth-promoting activity of LPA has been observed in numerous cell lines. The ability of serum to induce mitosis appears to be mediated, in large part, by the lipids LPA and SIP (22).

Endothelial cells are normally quiescent and form a tight monolayer by interacting with the underlying extracellular matrix (ECM) and surrounding endothelial cells (23). Interactions between endothelial cells and ECM proteins are important determinants of endothelial cell migration and signaling. In addition, endothelial cell migration is important during vascular development and angiogenesis. Both LPA and SIP stimulate endothelial cell migration (22, 24, 25). Matrix metalloproteinases (MMPs) have been reported to play critical roles in this process (26–28).

MMPs are a family of enzymes that degrade components of the ECM. They are grouped by their substrate preferences and also domain structures: collagennases (MMP-1, MMP-8, and MMP-13) degrade fibrillar collagen; gelatinases (MMP-2 and MMP-9) are potent in nonfibrillar and denatured collagen degradation; stromelysins (MMP-3, MMP-10, and MMP-11) prefer proteoglycans and glycoproteins as substrates; and membrane-type MMPs (MT1-, MT2-, MT3-, MT4-, and MT5-MMP) contain a C-terminal transmembrane domain directing cell surface localization (26, 29, 30).

The 72-kDa gelatinase A (MMP-2) is the most widely distributed of all the MMPs and is expressed constitutively by

BIOACTIVE LYSOPHOSPHOLIPIDS (LPLS), including lysophosphatidic acid (LPA) and sphingosine-1-phosphate (SIP), are membrane-derived lipid mediators, which are generated from phospholipid precursors of membranes and secreted by platelets (1, 2), macrophages, epithelial cells (3–5), and some cancer cells (6–8). These lysophospholipids regulate migration, proliferation, and survival of endothelial cells (9–11). LPA and SIP bind multiple G protein-coupled receptors of the endothelial differentiation gene (Edg) family (9, 12). To date, nine members of the family have been discovered in mammalian cells: LPA₁ (Edg-2), LPA₂ (Edg-4), LPA₃ (Edg-7), and LPA₄ are receptors for LPA (13, 14), and SIP₁ (Edg-1), SIP₂ (Edg-5), SIP₃ (Edg-3), SIP₄ (Edg-6), and SIP₅ (Edg-8) are receptors for SIP (9, 15). Activation of LPL receptors results in a wide spectrum of intracellular events, such as increases in inositol phosphates and intracellular calcium (9), inhibition of adenyl cyclase (16), and activation of kinases such as protein kinase C and many other signaling pathways (17–19).

The 72-kDa gelatinase A (MMP-2) is the most widely distributed of all the MMPs and is expressed constitutively by...
a number of cells, including endothelial cells. It participates in the breakdown of collagen type IV, a major component of subendothelial basement membranes (29). Therefore, MMP-2 production by endothelial or surrounding cells may be vital for the formation of new functional blood vessels, in either the early process of degradation or the later reconstruction of the underlying basement membrane. Paradoxically, at low levels, the specific physiological MMP-2 inhibitor, tissue inhibitor of metalloproteinases (TIMP)-2, promotes this activation by forming a membrane complex with MT1-MMP, anchoring the pro-MMP-2 to the cell surface (31–33). A recent study also showed that both TIMP-2 and TIMP-3 enhance the activation of pro-MMP-2 by MT3-MMP (34). It has been shown that MMP-2 and TIMP-2 are required for normal development of zebrafish embryos (35, 36), indicating that MMP-2 and TIMP-2 expressions are essential during embryogenesis.

Activities of MMPs are tightly regulated at several levels including gene expression and secretion of proenzymes that require activation by other MMPs or serine proteinases (37). Once MMPs are activated, they are subjected to inhibition by TIMPs, which determine the net enzymatic activity in the extracellular space (26). A balance between MMPs and TIMPs is involved in the morphogenesis of normal tissue as well as mechanisms of tumor invasion and metastasis (37–40).

Aberrant expression of MMPs has been noted in cancer, angiogenesis (41), arthritis, inflammation, emphysema, multiple sclerosis (42), and chronic wounds (6, 8). In this study, we hypothesized that LPA and S1P may affect endothelial cell invasion through regulating MMP-2 expression. We present evidence that LPA and S1P may affect endothelial cell invasion through regulating MMP-2 expression. We present evidence that LPA and S1P may enhance MMP-2 expression in human endothelial cell hybridoma EAHy926 cells in concentration- and time-dependent manner. By using chemical inhibitors, we show that LPA and S1P enhance MMP-2 through pathways dependent on MAPK kinase (MEK)/ERK, nuclear factor-kB (NF-kB), and calcium influx-mediated signaling. Furthermore, we used a modified Boyden chamber to investigate the invasive activity of EAHy926 cells and found that the invasive activity was enhanced by LPL treatments. This enhancement was likely due to an increase in MMP-2 expression in EAHy926 cells because the enhancement was prevented by pretreatment with the synthetic MMP inhibitor, GM6001. These results imply that LPL’s enhancement of endothelial cell invasion is likely mediated through induction of MMP-2 expression.

Materials and Methods

Reagents

SIP, oleoyl-LPA, and proteinase inhibitors, including leupeptin, sodium vanadate, phenylmethysulfonyl fluoride, and β-glycerophosphate, pertussis toxin, and pyrrolidinedithiocarbamate (PDTC) were purchased from Sigma (St. Louis, MO). Gelatin was obtained from United States Biochemical Corp. (Cleveland, OH). The chemical inhibitors, 1,27632 dihydrochloride, U73122, PD98059, and wortmannin, were purchased from Tocris Cookson (Ellisville, MO). 4-Amino-5-(4-chlorophenyl)-7-((butyl)pyrazol-3, 4-d) pyrimidine (PP2) was purchased from Calbiochem (San Diego, CA). Monoclonal mouse antibody against human MMP-2 was obtained from Santa Cruz Biotech (Santa Cruz, CA). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). Medium 199 (M199) and trypan blue stain were purchased from Life Technologies, Inc. (Gaithersburg, MD). TRizol, penicillin-streptomycin, and trypsin-EDTA were purchased from Invitrogen (Grand Island, NY). The MMP inhibitor, N-[2S]-2-(methoxy carbonyl-methyl)-4-methylpentanoyl-tryptophan-methylamide, GM6001, was purchased from Chemicon (Temecula, CA).

Cell culture

Human umbilical cords were kindly provided by Dr. S. M. Lee (Chun Shan Hospital, Taipei, Taiwan). Each umbilical cord was washed several times with 1× cross buffer [136.9 mM NaCl, 4 mM KCl, 10 mM HEPES, and 11.1 mM glucose (pH 7.65)] and then was incubated with 0.1% type IV collagenase (Sigma) in a 37 C incubator for 10 min. After incubation, the endothelial cells were washed out with serum-free M199 and cultured with 20% endothelial cell growth medium (Cell Applications Inc., San Diego, CA), in M199 with 5 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 1.0 mM sodium pyruvate, penicillin (100 IU/ml), streptomycin (100 IU/ml), and 10% FBS. Culture dishes were maintained in a humidified 5% CO2 at 37 C. After 16 h, nonadherent cells were removed, and the adherent cells were harvested. Experiments were performed with cells that had undergone no more than five passages.

Endothelial cell hybridoma EAHy926 cells were kindly provided by Dr. Cora-Jean S. Edgell (Department of Pathology, University of North Carolina, Chapel Hill, NC) (43). EAHy926 cells were cultured in M199 supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100 IU/ml), and glutamine (5 mM) in a humidified 5% CO2 at 37 C. Cells were cultured in six-well plates (Greiner Bio-One, Kremsmünster, Austria) for the experiments.

LPL stimulation

LPA and SIP were prepared in chloroform and methanol (1:9) and stored at −20 C. Endothelial cells were cultured to 70–80% confluence and starved in serum-free medium for 16–24 h. LPA and SIP treatments were prepared in serum-free M199 with 0.1% fatty acid-free BSA as a carrier.

RNA extraction, RT-PCR, cloning, and DNA sequencing

Total RNA from endothelial cells was isolated using the TRIzol reagent (Invitrogen), and 1 µg of total RNA was reverse-transcribed using reverse transcriptase enzyme (New England Biolabs, Beverly, MA). The generated cDNAs were subjected to PCR using primer sets for MMP-2 and GAPDH. Sequences of the oligonucleotide primers, and the expected size and annealing temperature for the reactions are summarized in Tables 1 and 2. PCR products were resolved on 1.5% agarose gel, stained with ethidium bromide, and photographed. All data were quantified with TotalLab software (version 2.01; Newcastle upon Tyne, UK). PCR product was extracted from agarose gel and cloned by TA-cloning kit (Invitrogen, Carlsbad, CA). Cloned cDNAs were sequenced by Mission Biotech Inc. (Taipei, Taiwan) to verify the identities of the sequences.

Real-time PCR

Real-time PCR was performed using the iCycler IQ real-time detection system (Bio-Rad Laboratories, Hercules, CA) with SYBR-green 1 (stock solution × 25,000, diluted at 1:1000) as the fluorescent dye enabling real-time detection of PCR products according to the manufacturer’s protocol. Gene-specific primers were used, and the specificity was tested under normal PCR conditions. The cDNA was subjected to real-time PCR using the primer pairs as given in Table 1. Cycling conditions were 95 C for 3 min, followed by 40 cycles of 94 C for 30 sec, 62 C for 30 sec, and 72 C for 60 sec. For quantification, the target gene was normalized to the internal standard GAPDH gene. Oligonucleotide primers for PCR were designed using Beacon Designer2 software (Premier Biosoft International, Palo Alto, CA).

Western blotting

Endothelial cell lysates were prepared as previously described. Briefly, control and LPA- or SIP-treated cells were washed with ice-cold water.

References
PBS twice and lysed with 200 μl of radioimmunoprecipitation assay buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% NaN3, 1 mM phenylmethylsulfonyl fluoride, and 2 mM β-glycerophosphate]. Lysates were collected with a cell scraper and clarified by centrifugation (10,000 × g for 10 min at 4°C). Protein concentrations were determined using the DC protein assay.

Equal amounts of protein (60 μg) were separated by 4–10% SDS-PAGE and then transferred to Immobilon-nitrocellulose transfer membranes (Millipore, Billerica, MA). Transferred blots were blocked with 5% BSA in TBS and Tween 20 for 2 h (room temperature) before incubation with mouse monoclonal antibody against human MMP-2 (1:1000 dilution; Santa Cruz Biotechnology). Membranes were then washed and incubated with horseradish peroxidase-conjugated goat antimouse antibody (1:2000). The blot was then incubated in ECL substrate (Santa Cruz Biotechnology) for 1 min at room temperature, and visualized by autoradiography.

Blots were stripped and reprobed with an antibody against human β-actin to demonstrate uniform loading of proteins. All data were quantified with TotalLab version 2.01 software.

**Gelatin zymography**

EAhy926 cells and human umbilical vein endothelial cells (HUVECs) were serum starved for at least 16 h before LPL treatment in serum-free medium. Conditioned media collected from both control (untreated) and LPL-treated cells were centrifuged at 5000 rpm for 10 min at 4°C. Anti-MMP-2 (1.500 dilution; Santa Cruz Biotechnology) were added to 0.5 ml of supernatants and incubated for 24 h at 4°C. The immunocomplexes were separated by incubating with 30 μl of Protein G Plus Protein A agarose suspension (Calbiochem) for 4 h at 4°C followed by centrifugation at 5000 rpm for 5 min at 4°C. The pellets were mixed with nonreducing sample buffer and processed for gelatin zymography.

**Invasion assay**

The invasive properties of endothelial cells were assayed in a modified Boyden chamber with a membrane filter. In brief, the upper surface of polycarbonate filters with 8-μm pores (Neuro Probe, Gaithersburg, MD) was precoated with 2% gelatin and dried at room temperature. The prepared filters (gelatin-coated filters) were extensively rehydrated with PBS before use. Confluent EAhy926 cells were serum-starved with serum-free M199 for 16 h. Cells were then trypsinized with trypsin-ENDTA, followed by incubation with M199 in the presence of 1% FBS to inhibit trypsin activity. Cell pellets were then resuspended in serum-free media to a concentration of 1 × 10⁶ cells/ml. Control and different-concentration LPL treatments were loaded in the lower chambers of the modified Boyden chamber as a chemotaxis inducer. Cells (2 × 10⁵ cells/ml at 50 μl per well) were plated in the upper chambers and allowed to invade through the 8-μm porous 2% gelatin-coated filters at 37°C for 4 h.

**TABLE 2. Human Edg receptor primer sets**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Product size (bp)</th>
<th>Temperature (C)</th>
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</thead>
<tbody>
<tr>
<td>edg1</td>
<td>S1P1</td>
<td>352</td>
<td>62</td>
</tr>
<tr>
<td>edg5</td>
<td>S1P2</td>
<td>512</td>
<td>62</td>
</tr>
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<td>edg3</td>
<td>S1P3</td>
<td>701</td>
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<tr>
<td>edg6</td>
<td>S1P4</td>
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<td>62</td>
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<tr>
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<td>S1P5</td>
<td>236</td>
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</tr>
<tr>
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<td>LPA1</td>
<td>397</td>
<td>62</td>
</tr>
<tr>
<td>edg4</td>
<td>LPA2</td>
<td>888</td>
<td>53</td>
</tr>
<tr>
<td>edg7</td>
<td>LPA3</td>
<td>392</td>
<td>62</td>
</tr>
</tbody>
</table>

**TABLE 1. Human primer sets**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>Temperature (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>Sense</td>
<td>TTC TAC AAT GAG CTG GGT GGC</td>
<td>417</td>
<td>62</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>GGT GGT CTC TCA GAG CAG CTT</td>
<td>215</td>
<td>60</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Sense</td>
<td>TTC CAG ACT TCA GCC TCT TGA GGG ACT GGT</td>
<td>493</td>
<td>60</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Antisense</td>
<td>GAP TGG ATG AGG AGG AGG</td>
<td>207</td>
<td>58</td>
</tr>
</tbody>
</table>

*Primer sets designed for real-time PCR.*
Noninvasive cells on the upper surface of the filter were wiped off with a cotton swab, and migrating cells adherent to the lower surface were fixed with 20% formaldehyde and stained with a 1% crystal violet solution in 5% ethanol. Cells that had invaded to the lower surface were quantified by colorimetric measurement using crystal violet staining.

Adhesion assay

The assays were modified and performed as previously described (44). Briefly, untreated polystyrene 96-well flat-bottom microtiter plates (Greiner Bio-One) were coated with 2% gelatin in the presence or absence of GM6001. Wells coated at 37°C for 1 h were washed with PBS [PBS: 137 mM NaCl, 2.7 mM KCl, and 10 mM Na2HPO4 (pH 7.4)]. EAhy926 cells were starved overnight in serum-free medium and then treated with control and 1 μM of LPA and S1P as indicated for 4 h. Cells were trypsinized and then coincubated with media in the presence or absence of 10 μM GM6001 in 1 h gelatin precoated 96 wells for 45 min. As a negative control, media alone were loaded into the wells. Nonadherent cells were vigorously washed by PBS three times. The attached cells were fixed and stained with a crystal violet solution [1% (wt/vol) crystal violet, 10% (vol/vol) ethanol] overnight at room temperature. Adherence was determined by absorption at 595 nm in a microplate reader (Bio-Rad Laboratories). The data were reported as the mean absorbance of triplicate wells ± SE minus the mean absorbance of media alone loaded wells.

GM6001 treatments

Cells were then trypsinized with trypsin-EDTA, followed by incubating with M199 in the presence of 1% PBS to inhibit trypsin activity. Cell pellets were then resuspended in serum-free media to a concentration of 1 × 10^5 cells/ml. The suspended cells were incubated in control medium or 10 μM GM6001, a broad MMP inhibitor, at 37°C for 1 h. Serum-free M199 control, 1 μM LPA, and 1 μM S1P treatments were loaded in the lower chambers of the modified Boyden chamber. Cells (5 × 10^4/well, in either control medium or 10 μM GM6001) were plated in the upper chambers and allowed to invade through the 8-μm porous filters coated with 2% gelatin for 4 h. Nonmigrating cells were removed, and the filter was fixed with 20% formaldehyde and stained with 1% crystal violet solution in 5% ethanol.

Chemical inhibition

A specific inhibitor of Gi (PTX, 15 ng/ml), a phospholipase C (PLC) inhibitor (U73122, 1 μM), a MEK/ERK inhibitor (PD98059, 10 μM), a phosphatidylinositol 3-kinase (PI3K) inhibitor (LY 294002, 20 μM), an NF-κB inhibitor (PDTC, 100 μM), a Src kinase inhibitor (PP2, 10 μM), and an inhibitor of receptor-mediated calcium entry (SKF 96365, 10 μM) were used in our assays to determine the signaling pathways involved. Cells were cultured to around 80% confluency and starved overnight in serum-free medium. All inhibitors were applied 1 h before LPL treatments.

Statistical analysis

Significant differences between control and treatment groups were tested using one-way ANOVA followed by Fisher’s protected least-significant difference test (StatView, Abacus Concept, Berkeley, CA) and two-way ANOVA followed by Duncan’s new multiple range test. Each experiment was repeated at least three times. A value of P < 0.05 was considered statistically significant.

Results

LPA and S1P enhance MMP-2 mRNA levels in human endothelial cells

We first determined the Edg receptor expression profile of EAhy926 cells by RT-PCR (Fig. 1). Our results indicated that these cells express Edg1, Edg5, LPA1, and LPA3 receptors and therefore can be used as a good model to study LPL’s effects in human endothelial cells.

Dose response.

LPLs have multiple effects in many different cell types, such as promoting cell migration, which requires protease. Therefore, we were interested in determining whether LPLs regulate MMP-2 expression in endothelial cells. We first investigated the effects of LPLs on MMP-2 mRNA levels in HUVECs. HUVECs were treated with 1 μM of LPA and S1P for 4 h. RNAs from treated cells were isolated and subjected to RT-PCR using specific primer sets for human MMP-2 and β-actin. MMP-2 mRNA levels were elevated after the treatments, especially after treatment with LPA (Fig. 2A, left panel). Expression patterns of β-actin, which was used as the loading control, did not significantly differ in either LPA- or S1P-treated samples (Fig. 2A, right panel). To further confirm that the observed bands were MMP-2 mRNA, PCR products from agarose gels were cloned and sequenced. Sequencing results verified the bands are human MMP-2.

Time course.

In the dose-response experiments, we showed that the enhancement of MMP-2 mRNA expression by both LPA and S1P in EAhy926 cells was concentration dependent. Therefore, we intended to investigate whether the effects of LPA and S1P on MMP-2 mRNA levels in EAhy926 cells were time dependent. EAhy926 cells were incubated with LPA (1 μM) and S1P (1 μM) for different time intervals, and RNA

![Fig. 1. Edg receptor expression profile in EAhy926 cells. cDNA of EAhy926 cells was analyzed by RT-PCR to determine the Edg receptor (LPL receptor) expression profile. GAPDH was used to confirm the cDNA quality. PCR products were separated by 1.5% agarose gel electrophoresis. The expected PCR products were 352, 371, 392, 236, and 215 bp for Edg 1, 2, 7, 8, and GAPDH, respectively. EAhy926 cells express Edg 1 and 8 (SIP 1 and 5) and Edg 2 and 7 (LPA 1 and 3). M, 100-bp marker; G, GAPDH.](image-url)
was subjected to RT-PCR using specific primer sets for human MMP-2 or β-actin. PCR products were separated by 1.5% agarose gel electrophoresis. The expected PCR products were 493 bp for MMP-2 and 417 bp for β-actin. EAhy926 cells were serum starved overnight and incubated with LPA or S1P for 4 h at various concentrations as indicated. EAhy926 cells were starved overnight and incubated with S1P (1 μM) and LPA (1 μM) for different time durations. RNA from treated cells was harvested and subjected to RT-PCR using specific primer sets for human MMP-2 or GAPDH. The reaction products were separated on a 1.5% agarose gel and photographed. A 100-bp DNA ladder was used as a marker. The expected PCR products were 207 bp for MMP-2 and 215 bp for GAPDH. Similar experiments were repeated at least three times, and a representative result is shown in the figure. Each bar of the histogram represents a minimum of three experiments, and data are presented as the mean ± SD. *Statistically different, compared with the level in control cells (P < 0.05).

**LPA and S1P enhance MMP-2 protein levels in human endothelial cells**

**Dose response.** Because the mRNA levels were increased by LPLs, we further investigated whether the elevated mRNA levels of MMP-2 were also correlated with protein expression levels. Cells were treated with different concentrations of LPA and S1P, and total protein was collected and detected by Western blot analysis. After a 4-h treatment, LPA and S1P both enhanced MMP-2 protein expression in concentration-dependent manners (Fig. 3A). Human β-actin Western blotting was used to show that the amount of sample loading did not significantly differ between control and treated samples. Consistent with the RT-PCR results, the enhancement effects of LPA and S1P on MMP-2 expression in EAhy926 cells peaked at 1 μM. For LPA treatment, MMP-2 protein expression appeared to peak at 0.5 μM and dropped at 5 μM, whereas for S1P, the peak was around 0.5–1 μM, then it dropped at 10 μM. Similar results were also observed in HUVECs (data not shown).

**Time course.** In the dose-response experiments, we showed that enhancement of MMP-2 protein levels by both LPA and
S1P in EAhy926 cells was concentration dependent. Therefore, we wanted to test whether the effects of LPA and S1P on MMP-2 protein expression were time dependent. EAhy926 cells were incubated with LPA (1 μM) and S1P (1 μM) for different time durations as indicated. Total cell lysates were collected, and MMP-2 protein expression levels were monitored by Western blotting using an anti-MMP-2 antibody. A human β-actin antibody was used to show that the amount of sample loading did not significantly differ between control and treated samples. Enhanced MMP-2 protein levels in EAhy926 cells were first observed as early as 1 h after treatment was initiated, peaked at around 4 h for S1P treatment and 4–8 h for LPA treatment, and declined thereafter. With overnight treatment, MMP-2 expression remained high in S1P-treated but returned to basal levels in LPA-treated samples. Therefore, we have shown that LPA and S1P up-regulate MMP-2 protein expression in EAhy926 cells in a time-dependent manner (Fig. 3B).

**LPA and S1P enhance MMP-2 enzymatic activity in human endothelial cells**

**Dose response.** Because protein levels were increased by LPLs, we further investigated whether the elevated protein levels of MMP-2 were also correlated with enzymatic activity. We next investigated the effects in both HUVECs and EAhy926 cells. Cells were treated with different concentrations of LPA and S1P, and conditioned medium was collected and resolved on a substrate gel (containing 0.1% gelatin). After staining, increased gelatinolytic activity was detected, and we found that LPLs induced MMP-2 activity in a concentration-dependent manner both in HUVECs (Fig. 4A) and EAhy926 cells (Fig. 4B). These results were consistent with the results of mRNA levels. HUVECs and EAhy926 cells showed similar results after LPL treatment in these assays. MMP-2 enzymatic activity first increased at 0.5 μM, and the activities increased with the concentration. The effect of S1P was more significant than that of LPA in regulating MMP-2 enzymatic activity. These results indicated that LPA and S1P induce endothelial cell proteolytic activity, which might stimulate the invasive capacity of endothelial cells. Incubation of the gel with EDTA significantly inhibited the enzymatic activity of the gelatinase, indicating that the responsible gelatinase was a metalloproteinase (data not shown).

**Time course.** In the dose-response experiments, we show that the enhancement of MMP-2 enzymatic activity by both LPA and S1P in EAhy926 cells was concentration dependent. We further investigated whether the effects of LPA and S1P on MMP-2 enzymatic activity were time dependent or due to starvation. Cells were simultaneously subjected to LPL treat-
ments or serum-free medium for different time durations. The conditioned media, including LPL-treated and control media, were collected and assayed on a substrate gel (containing 0.1% gelatin). LPLs induced MMP-2 activity in a time-dependent manner (Fig. 4C). The enhancement effects of LPA and S1P were first observed at 2 h after ligand treatment. Although control medium showed a slight increase in MMP-2 activity, LPL treatment generated a more-significant induction of MMP-2 enzymatic activity. Therefore, we subtracted the control value to quantify the increased multiples of MMP-2 activity stimulated by LPLs. It should be noted that in addition to starvation before treatment, cells in the overnight treatment were incubated in a serum-free condition for 48 h. Such a stressful situation may have caused a dramatic increase in cell secretion in general. That might be the reason that the conditioned medium collected in the overnight treatment showed significantly increased MMP-2 gelatinolytic activity.

Enhancement of MMP-2 gelatinolytic activity by LPL treatments is suppressed by GM6001 and 1, 10-O-phenanthroline. Because the LPLs showed enhancement effects on gelatinolytic activity in endothelial cells, we further confirmed whether LPLs enhance gelatinolytic activity by up-regulating MMP-2 enzymatic activity in endothelial cells. Cells were pretreated with GM6001 for 1 h, followed by control and 1 μM of LPA and S1P treatments for 4 h. Conditioned media were collected and resolved on a substrate gel. We found that LPL induction of MMP-2 enzymatic activity of the gelatinase was partially blocked by GM6001 (Fig. 5A). In addition, incubation of the gel with 1 mM of 1, 10-O-phenanthroline, a chelator of zinc (45), also significantly inhibited the enzymatic activity of MMP-2 (Fig. 5B), indicating that the responsible gelatinase was MMP-2. To further confirm that the observed bands in the 62-kDa gelatinolytic activities reflected MMP-2 activation, conditioned media harvested from EaHy926 cells were immunoprecipitated with the anti-MMP-2 antibody.
Consistent with the gelatin zymography results, conditioned media immunoprecipitated with the anti-MMP-2 antibody but not with normal mouse IgG generate a sharp band at the size of 62 kDa in gelatin zymography (Fig. 5C). These results confirmed LPA and S1P enhance gelatinolytic activity in human endothelial cells through up-regulating MMP-2 enzymatic activity.

LPLs effects on MMP-2 mRNA expression are involved in MEK/ERK-, NF-κB-, and calcium influx-dependent mechanisms. EAhy926 cells were pretreated with control media alone, 15 ng/ml of PTx (a Gi inhibitor), 10 μM of PP2 (an Src family kinase inhibitor), 20 μM of LY 294002 (a PI3K inhibitor), 100 μM of PDTC (an NF-κB inhibitor), 1 μM of U73122 (a PLC inhibitor), 10 μM of PD98059 (a MEK/ERK inhibitor), and 10 μM of SKF96365 (a calcium influx inhibitor) for 1 h. Inhibitor-treated cells were then treated with 1 μM of LPA or S1P for 4 h. MMP-2 mRNA expression levels were monitored by real-time PCR. Histograms represent quantification by real-time analysis. Multiple increases, compared with untreated cells, were shown. The average value for each sample was normalized with the amount of GAPDH. Each bar of the histogram represents a minimum of three experiments, and data are presented as the mean ± SD. These results were analyzed by two-way ANOVA. A level of $P < 0.01$ was accepted as significant. **, Statistically different compared with the level in control cells ($P < 0.01$).
LPLs effects on MMP-2 protein expression are involved in MEK/ERK-, NF-κB-, and calcium influx-dependent signaling pathways. Because the enhancement effects on mRNA levels by LPLs were blocked by the above mentioned chemical inhibitors, we further investigated whether these inhibitors also affected MMP-2 protein expression induced by LPLs. Cells were pretreated with the inhibitors mentioned above for 1 h, followed by control and 1 μM of LPA and S1P treatments for 4 h. Human β-actin Western blotting was used to indicate that sample loadings were even. Consistent with the mRNA results, we found that LPL induction of MMP-2 protein expression was blocked by PDTC, PD98059, and SKF96365 but not pertussis toxin. These results suggested that the enhancement effects of LPLs on MMP-2 protein expression are MEK, NF-κB, and calcium influx dependent but independent of the function of Gi (Fig. 7).

Enhancement of MMP-2 gelatinolytic activity by LPL treatments is partially suppressed by chemical inhibitors. Because the enhancement effects on mRNA and protein levels by LPLs were blocked by certain chemical inhibitors, we further investigated whether the effects of these inhibitors were also correlated with enzymatic activity. Cells were pretreated with the inhibitors mentioned above for 1 h, followed by control and 1 μM of LPA and S1P treatments for 4 h. Conditioned media were collected and resolved on a substrate gel. We found that LPL induction of MMP-2 activity was significantly blocked by SKF96365 but partially suppressed by PDTC and PD98059, implying that calcium mobilization, MEK/ERK, and NF-κB signaling were involved. (Fig. 8). These results are consistent with the above-mentioned mRNA and protein results.

LPA and S1P induce MMP-dependent endothelial cell invasion. We further investigated whether enhancements of MMP by LPLs were responsible for the cell invasive activity. We tested the hypothesis that LPL treatments induce endothelial cell invasion by an in vitro Boyden chamber invasion assay. No significant cell invasion was observed under control conditions, whereas dramatic enhancement was seen in the presence of LPLs. The enhancement effects occurred in a concentration-dependent manner (Fig. 9A). The maximal effects were about 4- and 5-fold increases by S1P and LPA treatments, respectively.

To further demonstrate that LPL-induced EAhy926 cell invasion was due to the induction of MMPs, we used GM6001, a chemically synthesized MMP inhibitor in the invasion experiments. We found that LPL-induced EAhy926 cell invasion was significantly inhibited by GM6001 (Fig. 9B). The amounts of invading cells were also monitored by microscopy (Fig. 9C). Together, these results suggested that the LPL-induced endothelial cell invasive capacity is MMP dependent.

GM6001 inhibited basal or LPL-induced endothelial cell adhesion to gelatin. Because endothelial cell invasion of gelatin gel was enhanced by LPLs through up-regulating MMP-2 enzymatic activity, we further investigated whether these enhancement effects were due to increase of cell adhesion. We tested the hypothesis by an in vitro adhesion assay. Treatments with 1 μM LPA or S1P had limited effects on

Eahy926 cell adhesion to gelatin gel. However, significant suppression of cell adhesion was observed in both control and treated Eahy926 cells coincubated with 10 μM GM6001 (Fig. 10). These results indicated that LPA and S1P strongly enhanced endothelial cell invasion but had limited effects on endothelial cell adhesion. On the other hand, GM6001, a broad MMP inhibitor, showed strong suppression effects on both endothelial cell invasion and adhesion. These results indicated that MMP-2 participates in both endothelial cell invasion of and adhesion to gelatin gel. In addition, the inhibitory effects of GM6001 on endothelial cell adhesion might also be responsible for the suppression of endothelial cell invasion induced by LPLs.
Discussion

Our current study provides evidence that LPA and S1P produced by some cancer cells or secreted by activated platelets might enhance MMP-2 expression in surrounding endothelial cells. The up-regulation of MMP-2 in human endothelial cells is directly correlated with its invasion potential. LPA levels in ovarian cancer patients are elevated in both plasma and ascites, suggesting that this bioactive lipid may contribute to early events that promote cancer cell dissemination. This idea was supported by the observation that LPA induces urokinase secretion by ovarian cancer cells (8, 46). MMP and urokinase-type plasminogen activator (uPA) systems are mutually related and are both involved in carcinoma progression through adjacent extracellular degradation. LPA and uPA are both present at elevated levels in ovarian cancer, and uPA has been reported to be linked to malignant transformation in ovarian cells (47). A previous study (48) also showed that uPA can activate several MMPs. Our preliminary data showed that LPLs also induce uPA activity by casein zymography assays (data not shown). These results strongly suggest that LPLs play critical roles in endothelial cell invasion.

Previous work suggested that S1P induces MMPs and integrin-dependent HUVEC invasion and lumen formation (49). In this report, we present the molecular mechanism of LPL regulation of MMP-2 expression in EAhy926 cells. Furthermore, the concentration- and time-dependent activation of MMP-2 by LPLs is also consistent with a receptor-mediated mechanism. Cell migration and proteolysis are two essential processes during tumor invasion and metastasis, and MMPs are prime candidates for these activities. Furthermore, there is accumulating evidence that elevated expression of
MMPs is observed in various cancer tissues (43, 50). It is now clearer that MMP substrates are not limited to the ECM but include other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor-binding proteins, cell surface receptors, and cell-cell adhesion molecules (39). MMPs are also known to solubilize cell surface and matrix-bound factors that can then act in an autocrine or paracrine manner to influence cellular properties such as growth, apoptosis, and migration. In prostate cancer cells, LPA-induced ERK activation involves MMPs, which cause the release of active epithelial growth factor receptor ligands and may induce cancer cell proliferation (51). This is an example of MMP-mediated transactivation of cell surface receptors by proteolytically cleavage of plasma membrane-anchored proteins to produce catalytically active hormones. Moreover, it has also been shown that LPA promotes MMP activation and MMP-dependent invasion in ovarian cancer cells (52). These results suggest that the effects of LPLs on cell invasion might be partially mediated through enhancement of MMP-2 expression.

LPA and S1P bind to Edg receptors and activate multiple G proteins including Gi, Gq, and G12/13. These activated G proteins, and the data are presented as the mean ± sd. These results were analyzed by two-way ANOVA. A level of $P < 0.05$ was accepted as significant. a, Significant differences between control and LPL treatment groups; b, significant differences between LPL treatment and inhibitor treatment groups. C, Amounts of invading cells were monitored by microscopy. a, Untreated control-1; b, S1P, 1 μM; c, S1P, 5 μM; d, untreated control-2; e, LPA, 1 μM; f, LPA, 5 μM; g, control pretreated with GM6001; h, LPA, 1 μM pretreated with GM6001; and i, S1P, 1 μM pretreated with GM6001. Similar experiments were repeated three times, and a representative result is shown in the figure. Bar, 300 μm.

Fig. 9. LPA and S1P increase invasion of EAhy926 cells, and the effect is blocked by the MMP inhibitor. EAhy926 cells were starved overnight in serum-free medium. A, Control and 0.5, 1, and 5 μM of S1P and LPA treatments were loaded in the lower chamber as a chemotaxis inducer. Cells at $5 \times 10^4$/well were plated in the upper chamber and allowed to migrate through 8-μm porous filters coated with 2% gelatin for 4 h. Noninvading cells were removed, and the filter was fixed and stained with crystal violet. B, Suspended cells were pretreated with GM6001, a broad MMP inhibitor, for 1 h. GM6001 significantly suppressed endothelial cell invasion enhanced by LPLs. Each bar of the histogram represents a minimum of three experiments, and the data are presented as the mean ± sd. These results were analyzed by two-way ANOVA. A level of $P < 0.05$ was accepted as significant. a, Significant differences between control and LPL treatment groups; b, significant differences between LPL treatment and inhibitor treatment groups. C, Amounts of invading cells were monitored by microscopy. a, Untreated control-1; b, S1P, 1 μM; c, S1P, 5 μM; d, untreated control-2; e, LPA, 1 μM; f, LPA, 5 μM; g, control pretreated with GM6001; h, LPA, 1 μM pretreated with GM6001; and i, S1P, 1 μM pretreated with GM6001. Similar experiments were repeated three times, and a representative result is shown in the figure. Bar, 300 μm.

Fig. 10. GM6001 blocked basal or LPL induced-endothelial cell adhesion to gelatin. EAhy926 cells were starved overnight in serum-free medium and then treated with control and 1 μM of LPA and S1P as indicated for 4 h. Cells were trypsinized and then coincubated with media in the presence (open bars) or absence (solid bars) of 10 μM GM6001 in gelatin precoated 96-wells for 45 min. Adhered EAhy926 cells were washed and stained by crystal violet and quantified by OD595. Histograms represent quantification results of adherent EAhy926 cells. Similar experiments were repeated three times, and a representative result is shown in the figure. * Statistically different, compared with the level in control cells ($P < 0.05$).
proteins turn on downstream signaling events including MAPK phosphorylation, Ca^{2+} mobilization, and cytoskeleton reorganization (24). Herein, we report that the effect of LPA and S1P on endothelial cell invasion activity can be inhibited by MEK/ERK, NF-κB, and calcium influx inhibitors (Fig. 8). MMP-13 induction in human osteoblastic cells and the IL-1β- and TNFα-induced MMP-1 secretion in human pancreatic cell are involved in the MEK/ERK pathway (53). Additionally, calcium influx has been suggested to modulate expression of MMP-2 (54). Collectively, these results suggest that the effects of LPA and S1P on endothelial cell MMP-2 expression are mediated by Edg receptors and multiple G protein-activated downstream effectors.

The enhancement effects of MMP-2 gelatinolytic activity by LPA and S1P were only partially suppressed by PD98059, PDTC, and SKF96365. This was possibly due to the fact that the enzymatic activity of MMP-2 was regulated by a complicated and delicate mechanism, including posttranscription, secretion regulation, proteolytic activation, and proteolytic inhibition. Therefore, gelatinolytic activities do not reveal complete inhibition effects as those on mRNA levels. Another possible reason is that the quantification of gelatin zymographic analyses is not as precise.

MMPs are up-regulated in many types of human cancer, and their expression is often associated with poor survival (55). Several studies have reported the up-regulation of MMP-2 in cancer cells, including breast, colon, ovarian, pancreatic, and liver cancers (37, 56). Whereas some MMPs (for example, MMP-1, MMP-2, and MMP-7) are expressed by cancer cells, other MMPs (for example, MMP-2 and MMP-9) are also synthesized by tumor stromal cells, including fibroblasts, myofibroblasts, inflammatory cells, and endothelial cells. Animal studies have also shown that in prostate cancers, only cells expressing high levels of MMP-2 and MMP-9 have the ability to metastasize (57). Metastasis is a major cause of death among cancer patients, which requires several sequential events, such as changes in cell-ECM interactions, the disconnection of intercellular adhesion and separation of single cells from solid tumor tissue, degradation of the ECM, and the movement of tumor cells into the ECM. Relatively benign cells acquire malignant properties when MMP activity is increased or TIMP activity diminished (58, 59). This is consistent with our findings that the enhancement of MMP-2 is related to the invasive properties of endothelial cells, suggesting that LPLs might contribute to cancer invasion by enhancement of MMP-2 production from an endothelial origin. MMP-2-deficient mice showed slightly smaller body weight but displayed significant reduction in both the primary tumor mass and metastatic spread (60). MMP-2 (and other MMPs as well) therefore are tempting targets for therapeutic intervention strategies in diseases such as cancer. The phenotypes of some MMP-deficient mice suggest, however, that this approach may give rise to severe side effects. MMP inhibitor therapy has yielded spectacular results in animal models, but unfortunately not in humans (61).

During angiogenesis, endothelial cells experience loosening of the matrix and intercellular adhesion; degradation of the subendothelial matrix; and the migration, proliferation, and formation of new tubes (62–64). MMPs play an important role in these steps by degrading the ECM so that cells can move across tissues into nearby stroma (65). SIP-induced invasion may facilitate the guidance of endothelial cells creating an initial sprouting and may also contribute to the ability of endothelial cells (49). These results are consistent with our current observation that LPLs might be important regulators for endothelial cells by modulating MMP-2 expression.

The broad-spectrum MMP inhibitor, GM 6001 (galardin, ilomastat), is a hydroxamic acid originally synthesized as an inhibitor of human skin collagenase, and has been shown to block MMP-1, MMP-2, MMP-3, and MMP-9 (66). In our study, we showed that LPL-induced human endothelial cells invasion was blocked by this inhibitor. Using trypan blue staining, which distinguishes viable from nonviable cells, we confirmed that GM6001’s inhibition of endothelial invasion was not due to the reagent having a lethal effect at the indicated concentration (data not shown). Koike et al. (67) group observed that aged human microvascular endothelial cell lines exhibited poor formation of tubular, capillary-like structures in vitro, and diminished expression of active MMP-2 and also showed that GM6001 decreased tubulogenesis in endothelial cells. Another study showed that GM6001 blocked LPA-induced epithelial growth factor receptor transactivation in rat-1 cells (68). Moreover, Bayless’ group used GM6001 to inhibit SIP-induced MMP-2 activation (49). Those results are consistent with our current observations.

We found that both LPA and S1P up-regulate MMP-2 protein expression levels as little as 1 h. Previous studies indicated that some proinflammatory cytokines such as TNF-induced MMP-9 expression through modulating inhibitory κB phosphorylation, ubiquitination, and degradation (69). These results suggest that LPLs might induce MMP-2 expression through these processes and result in rapid changes of MMP-2 protein expression.

In summary, our study clearly indicates that LPLs increase MMP-2 mRNA, protein, and enzymatic activities in concentration- and time-dependent manners, and these enhancements are consistent with a receptor-mediated mechanism. The induction is mediated by MEK/ERK-, NF-κB-, and calcium influx-dependent signaling pathways. Furthermore, the enhancement effects of LPA and S1P on MMP-2 expression in EAhy926 cells are responsible for the induction of the invasive activity. These results suggest that LPA and S1P might be important regulators of the interaction of cells and the extracellular matrix. Both lipids might play critical roles in regulating angiogenesis and cancer metastasis through the regulation of MMP-2.

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 References


36. Wu et al. • LPLs Induce MMP-2 in Endothelial Cells

Endocrinology, August 2005, 146(8):3387–3400
64. Langlois S, Gingras D, Beliveau R 2004 Membrane type 1-matrix metalloproteinase (MT1-MMP) cooperates with sphingosine 1-phosphate to induce endothelial cell migration and morphogenic differentiation. Blood 103:3020–3028

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