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Lysophospholipids Increase IL-8 and MCP-1 Expressions in Human Umbilical Cord Vein Endothelial Cells Through an IL-1-Dependent Mechanism

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Abstract Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are both low-molecular-weight lysophospholipid (LPL) ligands which are recognized by the Edg family of G protein-coupled receptors (GPCRs). In endothelial cells, these two ligands activate Edg receptors resulting in cell proliferation and cell migration. Interleukin-8 (IL-8) is a C-X-C chemokine and acts as a chemoattractant of neutrophils, whereas monocyte chemoattractant protein-1 (MCP-1) is a C-C chemokine and functions mainly as a chemoattractant of monocytes/macrophages. Both factors are secreted from endothelial cells and have been implicated in the processes leading to atherosclerosis. We examined the effects of LPLs on the expression of IL-8 and MCP-1, key regulators of leukocyte recruitment in human umbilical cord vein endothelial cells (HUVECs). Work illustrated in this article showed that LPA and S1P enhanced IL-8 and MCP-1 mRNA expressions, and protein secretions in dose- and time-dependent fashions. Maximal mRNA expression appeared at 16 hr post-ligand treatment. Using prior treatments with chemical inhibitors, LPLs enhanced IL-8 and MCP-1 expressions through a Gi-, Rho-, and NFκB-dependent mechanism. In a chemotaxis assay system, LPL treatments of endothelial cells enhanced monocyte recruitment through upregulating IL-8 and MCP-1 protein secretions. Pre-incubation with AF12198, an IL-1 receptor antagonist or IL-1 functional blocking antibody both suppressed the enhanced effects elicited by LPLs of IL-8 and MCP-1 mRNA expressions in HUVECs. These results suggest that LPLs released by activated platelets might enhance the IL-8- and MCP-1-dependent chemoattraction of monocytes toward the endothelium through an IL-1-dependent mechanism, which may play an important role in facilitating wound-healing and inflammation processes. J. Cell. Biochem. 99: 1216–1232, 2006.

Key words: LPA; S1P; IL-8; MCP-1; endothelial cells

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are potent phospholipid mediators with diverse biological activities [Moolenaar, 1995; Goetzl and An, 1998]. Concentrations of LPA and S1P reach micromolar levels in serum, and in some circumstances in plasma and account for some cellular growth effects of serum [Eichholtz et al., 1993; Tokumura, 1995; Yatomi et al., 1997; Goetzl et al., 2002]. The major sources of these two hydrolytic phospholipids are mainly activated platelets, injured cells, cells stimulated by

Abbrevation used: LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; LPL, lysophospholipid; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-l; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; Edg, endothelium differentiation gene; GPCR, G protein-coupled receptor; $[Ca^{++}]_i$, intracellular calcium concentration; PTX, pertussis toxin; exoC3, C3 exoenzyme; PDTC, pyrrolidine dithiocarbamate; NF-κB, nuclear factor-kappa B; HUVEC, human umbilical cord endothelial cell; BSA, bovine serum albumin.

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protein cytokines, and growth factors [Eichholtz et al., 1993; Moolenaar, 1995], and certain kinds of cancer cells such as ovarian cancer cells but not breast cancer or leukemia cells [Shen et al., 1998; Xu et al., 1998].

The cellular effects of LPA and S1P are mediated by two subfamilies of G protein-coupled receptors (GPCRs) encoded by endothelial differentiation genes (Edg Rs) [An et al., 1998; Chun et al., 1999; Hla et al., 1999; Moolenaar, 1999; Spiegel and Milstien, 2000]. Human Edg1 (S1P1), Edg3 (S1P3), Edg5 (S1P2), Edg6 (S1P4), and Edg8 (S1P5) transduce signals from S1P. Human Edg2 (LPA1), Edg4 (LPA2), and Edg7 (LPA3) transduce signals from LPA. G protein-linked intracellular signaling pathways activated by cloned Edg receptors, which have been characterized in heterologous expression systems, include ras-dependent activation of Erk 1/2, increases in $[Ca^{++}]_i$, and recruitment of rho GTPase and its downstream targets [Moolenaar, 1995; An et al., 1998; Chun et al., 1999; Hla et al., 1999; Spiegel and Milstien, 2000]. Endothelial cells form the inner lining of blood vessels and participate in important physiological processes including materials exchange, coagulation, and wound healing. Several pathological phenotypes, including arteriosclerosis, inflammation, and cancer, are associated with excessive activation or abnormalities of endothelial cells [Folkman and Shing, 1992]. Endothelial cells express at least seven types of Edg receptors, including Edg1 to Edg7 [Muehlich et al., 2004]. LPA and S1P regulate several endothelial functions through Edg Rs, including proliferation, migration, and secretion of proteases [Panetti et al., 1997, 2001; Wang et al., 1999; Wu et al., 2005]. It has also been suggested that these lipids might also play a role in the regulation of angiogenesis and blood vessel integrity [Lee et al., 1999a,b; Liu et al., 2000].

Extravasation of blood leukocytes into inflamed tissue is a crucial step in the development of inflammation and atherosclerosis [Springer, 1994]. The expression of adhesion molecules on the endothelium initiates the accumulation of leukocytes on the luminal surface of the vascular endothelium [Springer, 1990]. Subsequently, leukocytes actively migrate across the endothelial monolayer into the interstitium, a process referred to as transendothelial migration or diapedesis [Luscinskas et al., 1994; Springer, 1994]. The movement of specific populations of leukocytes into inflamed tissue is thought to be mediated by concentration gradients of chemokines that appear to be chemotactic for specific groups of leukocytes. Therefore, the extravasation of leukocytes into inflamed tissue relies on coordination of adhesion molecules expressed by the endothelium, leukocyte adherence, and specific chemotactic gradients. Members of the chemokine family have mostly been classified into two broad groups based on the juxtaposition of the first two cysteine residues in their amino acid sequences. The C-X-C family, typified by interleukin-8 (IL-8), is primarily chemotactic for neutrophils, while the C-C chemokine family, typified by monocyte chemoattractant protein-l (MCP-l), is primarily chemotactic for mononuclear leukocytes [DeVries et al., 1999; Scapini et al., 2000].

Previous studies indicated that IL-8 and monocyte chemoattractant protein-l (MCP-1) are involved in the entire process of leukocyte transmigration into tissues [Weber et al., 1999]. IL-8 and MCP-1 stimulate the chemotaxis of monocytes and are produced by monocytes and nonimmune cells such as fibroblasts, keratinocytes, and endothelial cells. In particular, the accumulation of IL-8 and MCP-1 at the luminal surface of the endothelium establishes an IL-8 and MCP-1 gradient across the endothelial monolayer, thereby potentiating the transendothelial migration of monocytes [Baggiolini et al., 1997].

Expressions of IL-8 and MCP-1 in endothelial cells can be upregulated by proinflammatory mediators such as IL-1β, TNFα [van den Berg et al., 1998], and lipopolysaccharide [Luster, 1998], depending on the activity of nuclear factor-kappa B (NF-κB), a transcription factor [Schlondorff et al., 1997], and also by plasma lipoproteins such as oxidized LDLs [Rosenson, 2004]. Since LPA and S1P stimulate the expression of these inflammatory cytokines from macrophages [Lee et al., 2002], and LPA is generated along with oxidized LDLs [Siess et al., 1999; Essler et al., 2000; Maschberger et al., 2000], we hypothesized that LPA and S1P may affect endothelial cell interactions with leukocytes through modulating the expressions of IL-8 and MCP-1. In this report, we present evidence that both LPA and S1P enhance IL-8 and MCP-1 expressions in human umbilical cord endothelial cell (HUVECs) in a time- and concentration-dependent fashion. Further-

more, by using chemical inhibitors, we show that both LPA and S1P upregulate IL-8 and MCP-1 expressions through a Gi/o-, Rho-, and NFκB-dependent mechanism, which is consistent with the signaling pathways activated by LPLs binding to Edg receptors. In addition, chemotactic activity of endothelial cells is enhanced by LPL treatment and significantly inhibited by pre-incubation with the functional blocking antibody against human IL-8 and MCP-1. Furthermore, we also demonstrate that LPLs upregulate IL-8 and MCP-1 mRNA expressions through an IL-1-dependent mechanism. These results imply that the inflammatory effects of LPLs are likely mediated through enhancement of the expressions of IL-8 and MCP-1, which contribute to monocyte recruitment.

MATERIALS AND METHODS

Reagents and Antibodies

Sphingosine 1-phosphate (S1P) was purchased from Biomol (Plymouth, PA). 1-Oleoyllysophosphatidic acid (LPA), fatty acid-free bovine serum albumin (FAF-BSA), pertussis toxin (PTX), and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma (St. Louis, MO). The C3 exoenzyme (exoC3) was purchased from List Biological Laboratory (Campbell, CA). AF12198 was purchased from Tocris (Ballwin, MO). The recombinant human tumor necrosis factor (TNF-α) was purchased from R&D Systems (Minneapolis, MN). The functional blocking anti-human IL-8 polyclonal antibody (ab7747) was purchased from Abcam (Cambridge, UK), the functional blocking anti-human MCP-1 monoclonal antibody (clone 5D3-F7) was purchased from Pharmingen (San Diego, CA), and the functional blocking anti-human IL-1 monoclonal antibody (clone 8516) was purchased from R&D Systems. Fetal bovine serum (FBS) and M199 were purchased from Hyclone (Logan, UT). RPMI-1640 medium and trypsin were purchased from Gibco BRL (Grand Island, NY). Endothelial cell growth medium (EGM) was purchased from Cell Applications (San Diego, CA). Penicillin, streptomycin, and L-glutamine were purchased from Invitrogen (Carlsbad, CA).

Cell Culture

HUVECs were isolated from fresh umbilical cords by treatment with 1% (v/v) collagenase in PBS at 37°C for 10 min. After elution with M199 containing 20% FCS, HUVECs were cultured on 0.04% gelatin-coated (Sigma) 10-cm plates in M199 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine (Invitrogen), 10% (v/v) FBS, and 25% (v/v) EGM, and cells underwent one passage weekly. Cells were subcultured after trypsinization (0.5% (v/v) trypsin solution, supplemented with 0.2% (v/v) EDTA) and used throughout passages two to four. The human leukemic monoblast cell line, and the human acute monocytic leukemia cell line, THP-1 (TIB202), were purchased from ATCC (American Type Culture Collection, Manassas, VA) and cultured in RPMI-1640 medium (supplemented with 0.2% (w/v) $NaHCO_3$ and 0.03% (w/v) L-glutamine) containing 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 10% (v/v) of FBS. Unless otherwise indicated, all cultures were grown at 37°C in a humidified atmosphere containing 5% CO_2 in 175-cm^2 culture flasks.

RT-PCR

Total cellular RNA was extracted from HUVECs using the TRIzol reagent (Gibco), and a Superscript kit (Gibco) was used for the reverse-transcription (RT) synthesis of cDNA. Sequences of the oligonucleotide primers, and the expected size and annealing temperature for the reactions are summarized in Table I. PCR products were resolved on 2% agarose gels,

TABLE I. Human Primer Sets

Primer		Sequence	Product size (bp)	Temp. (°C)
IL-8	Sense	ATG ACT TCC AAG CTG GCC GTG GCT	267	55
	Antisense	TCT CAG CCC TCT TCA AAA ACT TCT C		
MCP-1	Sense	TCT CAG TGC AGA GGC TCG CGA	162	55
	Antisense	GAG TGA GTG TTC AAG TCT TCG		
IL-1β	Sense	AAA CAG ATG AAG TGC TCC TTC AGG	390	55
	Antisense	TGG AGA ACA CCA CTT GTT GCT CCA		
GAPDH	Sense	ACC ACA GTT CAT GCC ATC AC	450	55
	Antisense	TCC ACC ACC CTG TTG CTG TA		

stained with ethidium bromide, and photographed.

Semiquantitative RT-PCR

To assess the levels of ICAM-1 mRNA in HUVECs, RT-PCR results were scanned using a PhosphoreImager® and analyzed by ImageQuaNT® software (Amersham Biosciences, Piscataway, NJ). Human GAPDH from the same sample as for the RT-PCR was used as an internal control to correct the loading.

ELISA

The IL-8 and MCP-1 concentrations in the culture supernatant were measured by ELISA kits purchased from BioSource (Carmarillo, CA) and Peprotech (Rocky Hill, NJ), respectively. An antibody specific for the cytokine to be studied was coated onto the wells of the 96-well ELISA plate. Samples, including standards with known cytokine contents, control specimens, and unknowns, were pipetted into these wells, followed by the addition of a biotinylated second antibody. During the first incubation, the cytokine antigen simultaneously bound to the captured antibody on one site and to the solution phase biotinylated antibody on a second site. After removal of excess second antibody, conjugated streptavidin peroxidase (Chemicon International, Temecula, CA) was added. This enzyme bound to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all of the unbound enzyme, a substrate solute was added, which was acted upon by the bound enzyme to produce color. The intensity of this colored product was directly proportional to the concentration of cytokines present in the original specimen.

Chemotaxis Assays

The trans-well migration of human THP-1 cells toward conditioned media with or without LPL treatment was studied in a 48-well microchamber (Neuro Probe, Cabin John, MD) as described previously [Ben-Baruch et al., 1995]. Conditioned medium (27 μl) was placed in the lower wells of the chamber and THP-1 cells (50 μl, 5×10^6/ml) were loaded in the upper wells. The lower and upper wells were separated by a nitrocellulose filters with an 8-μm pore size (Neuro Probe). The chamber was incubated for 4 hr at 37°C in humidified air with 5% CO_2. At the end of the incubation period, the chamber was disassembled and the cells remaining on the upper surface of the filter were removed by scraping the filter with a rubber scraper. The filters were then fixed and stained with a crystal violet solution (1% (w/v) crystal violet and 10% (v/v) ethanol). Blue spots developed in positions at which cell migration had occurred. Cells migrating across the filter onto the lower surface were photographed using light microscopy. Quantification of the migration results were determined by the color intensity of the spots. Samples on filters were solubilized in solubilization buffer (50% (v/v) 0.1 M NaH_2PO_4 (pH 4.5) and 50% (v/v) ethanol) overnight at room temperature, and the absorbance was determined at 540 nm on a microplate reader. All data are the relative expressions compared to untreated cells.

Statistical Analysis

Significant differences between treatments were tested using ANOVA followed by Duncan's new multiple range test (StatView; Abacus Concept, Berkeley, CA). Each experiment was repeated at least three times. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Both LPA and S1P Increase IL-8 and MCP-1 mRNA in HUVECs

Previous study observed that several genes which participate in inflammatory processes are upregulated by both LPA and S1P treatments [Graler and Goetzl, 2002]. To further confirm these observations, we determined the expressions of IL-8 and MCP-1 mRNA in LPL-treated HUVECs by RT-PCR. As shown in the center panels of Figures 1 and 2, IL-8 and MCP-1 mRNA levels increased in both LPA- and S1P-treated samples in concentration- (Fig. 1A,B) and time (Fig. 2A,B)-dependent manners. The expression patterns of GAPDH, which was used as the loading control, did not significantly differ in either LPA- or S1P-treated samples (Figs. 1 and 2, bottom panels). The RT-PCR results were quantified (Figs. 1 and 2, upper panels). The stimulatory effects of IL-8 and MCP-1 by both LPA and S1P treatments were first observed with treatment at 0.5 μM. Subsequently, LPA induced IL-8 and MCP-1 mRNA expression 5.4- and 2.8-fold at 5 μM, gradually

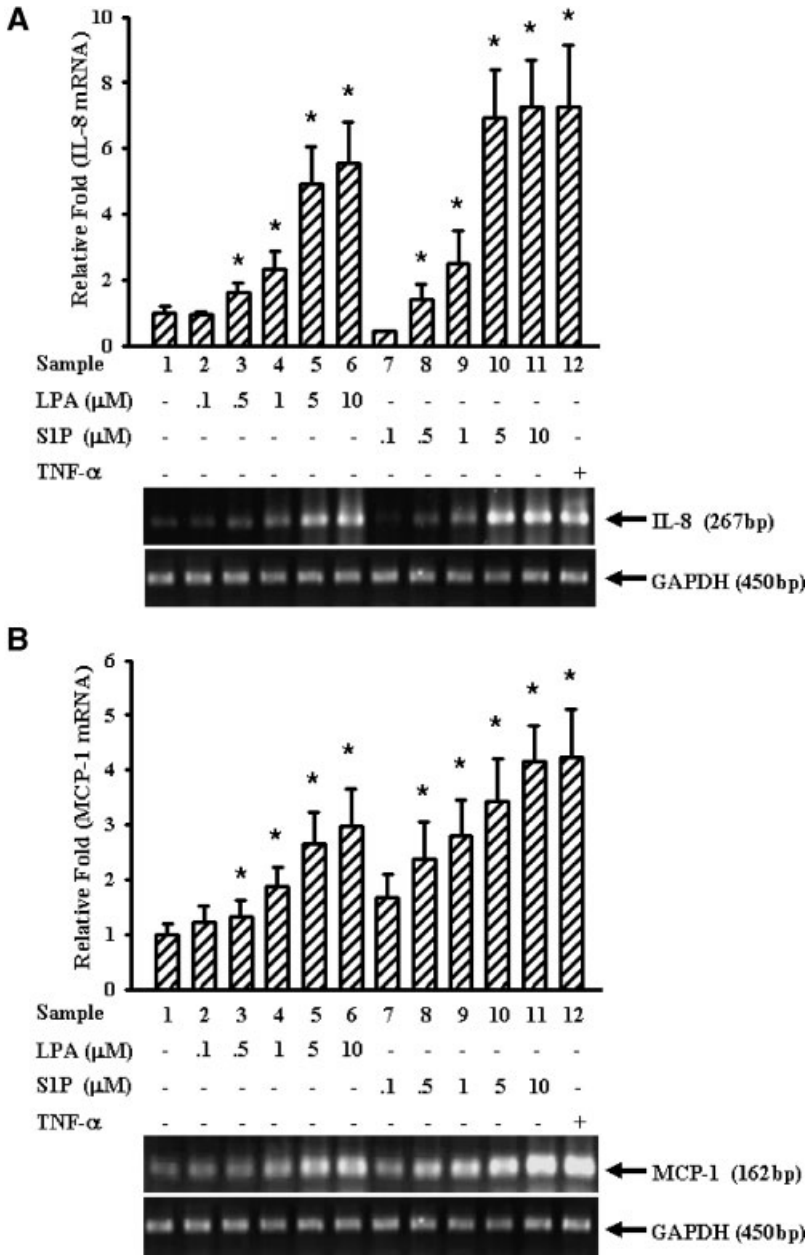

Fig. 1. LPA and S1P upregulate IL-8 and MCP-1 mRNA expressions in HUVECs in concentration-dependent manners. HUVECs were incubated with LPA or S1P for 16 hr at various concentrations as indicated. RNAs from treated cells were harvested and subjected to RT-PCRs using specific primer sets for human IL-8 (**A**) and MCP-1 (**B**) or GAPDH. The reaction products were separated on 2% agarose gels and photographed. TNF-α was used as the positive control. Similar experiments were repeated three times, and a representative result is shown in the figure. Histograms represent quantification of RT-PCR corrected with GAPDH and analyzed by PhosphorImager® using ImageQuaNT® software. $^*P < 0.05$ compared to the control. All data are relative multiples of expression compared to untreated cells. The data are representative of three experiments and are expressed as the mean ± SE.

reached 5.7- and 3-fold at 10 μM. S1P induced IL-8 and MCP-1 mRNA expression 7.7- and 4-fold at 5 μM, gradually reached 7.8- and 4.1-fold at 10 μM. Significant increases ($P < 0.05$) in both IL-8 and MCP-1 mRNA levels were first observed as early as 2 hr after the LPA or S1P were added, peaked at 16 hr, and decreased thereafter. These results indicated that both LPA and S1P enhance IL-8 and MCP-1 mRNA expressions in HUVECs.

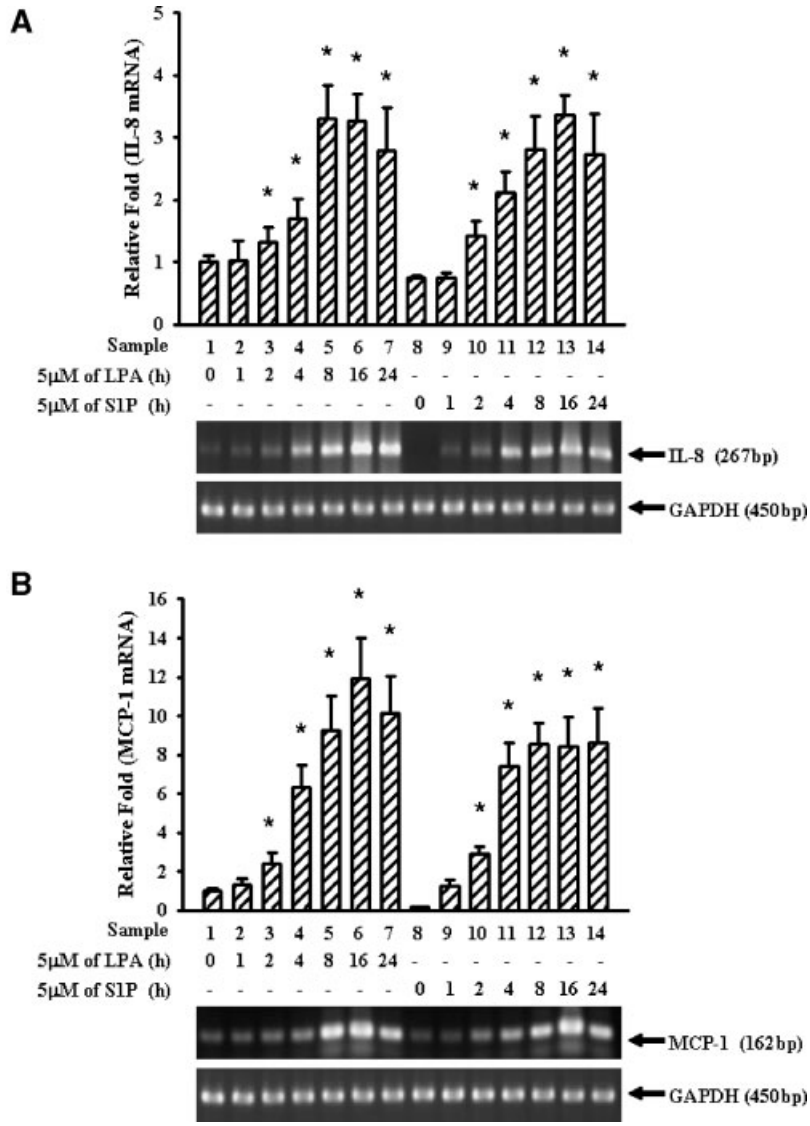

Fig. 2. LPA and S1P upregulate IL-8 and MCP-1 mRNA expressions in HUVECs in time-dependent manners. HUVECs were incubated with LPA (5 μM) or S1P (5 μM) for various times as indicated. RNAs from treated cells were harvested and subjected to RT-PCRs using specific primer sets for human IL-8 (**A**) and MCP-1 (**B**), or GAPDH. Similar experiments were repeated three times, and a representative result is shown in the figure. Histograms represent quantification of RT-PCR corrected with GAPDH analyzed by PhosphoreImager® using ImageQuaNT® software. $^*P < 0.05$ compared to 0 hr treatment. All data are relative multiples of expression compared with untreated cells. The data are representative of three experiments and are expressed as the mean ± SE.

LPA and S1P Increase IL-8 and MCP-1 Protein Expressions

Since mRNA levels were increased by LPLs, we further investigated if the elevated mRNA levels of IL-8 and MCP-1 were also correlated with protein expression levels. The total IL-8 and MCP-1 protein levels in LPL-treated cells were detected using ELISA. Both LPA and S1P enhanced IL-8 and MCP-1 protein expressions in concentration- (Fig. 3A,B) and time- (Fig. 3C,D) dependent manners. Consistent with the RT-PCR results, the enhancement effects of LPA and S1P on IL-8 and MCP-1 protein expressions in HUVECs peaked at a concentration of 10 μM. In the time-course experiments, the enhancement effects of both LPA and S1P (Fig. 3C,D) on IL-8 and MCP-1 protein expressions peaked at 24 hr after treatment. These results indicated that the

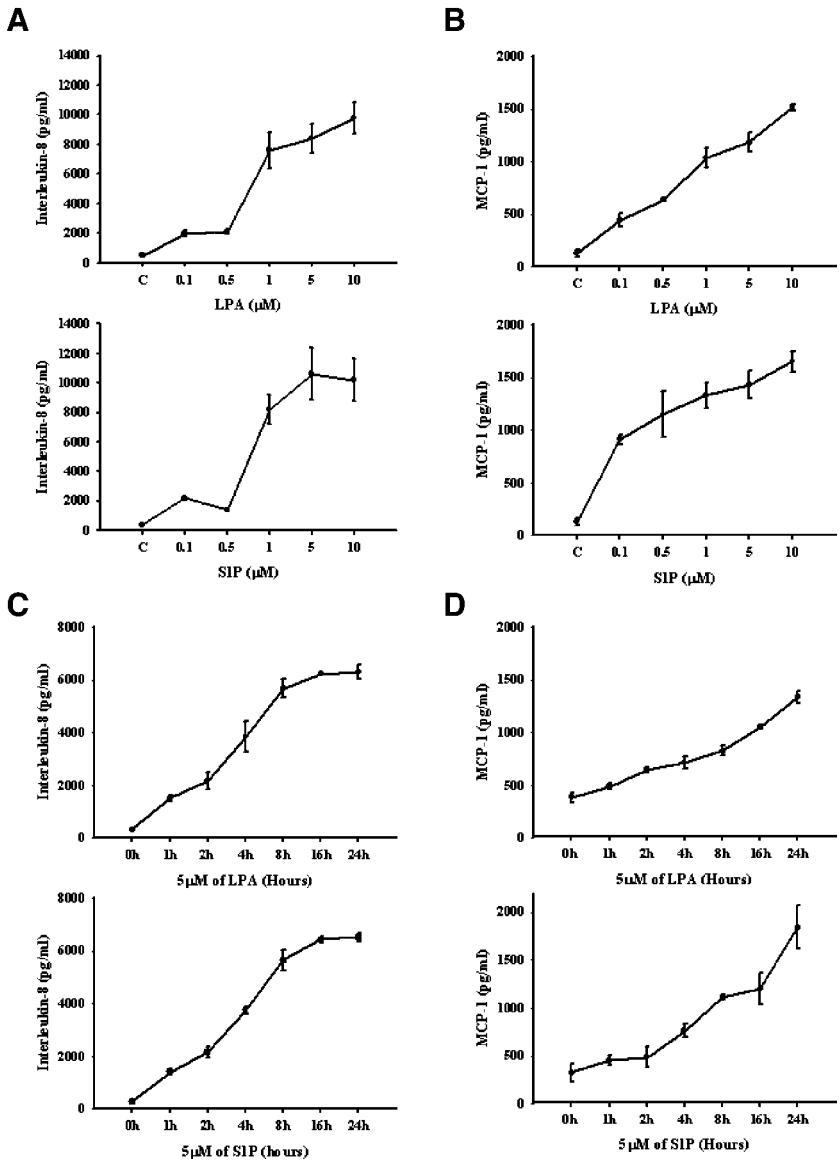

Fig. 3. LPA and S1P upregulate IL-8, and MCP-1 protein expressions in HUVECs in concentration- and time-dependent manners. Levels of IL-8 (**A**) and MCP-1 (**B**) in supernatant of cultured HUVECs were measured by ELISAs after incubation with LPA or S1P for 24 hr at various concentrations as indicated. Patterns of IL-8 (**C**) and MCP-1 (**D**) protein expressions were determined after HUVECs were treated with 5 μM of LPA or S1P for various times as indicated. Subpart C represents the untreated control. All data are expressed as the mean ± SE. Similar experiments were repeated three times, and a representative result is shown in the figure.

enhancement effects of LPLs on IL-8 and MCP-1 protein expressions might increase the chemotactic activities of endothelial cells toward leukocyte recruitment during wound-healing and inflammatory processes.

PTx, exoC3, and PDTC Blocked LPA and S1P Effects on IL-8 and MCP-1 Expressions in HUVECs

Since LPA and S1P are ligands for Edg receptors, and at least seven different Edg receptors are expressed on HUVECs [Muehlich et al., 2004], we further investigated if the effects of LPLs on IL-8 and MCP-1 expressions are mediated through these receptors. We addressed this question by using chemical inhibitors known to impede signaling processes of activated Edg receptors. PTX, a specific inhibitor of $G_{i/o}$ proteins, which has been shown to inhibit $G_{i/o}$-dependent LPL effects in different cell systems including HUVECs [Lee et al.,

1999b], was used in our assay. Pretreatment with 10 ng/ml of PTx for 12 hr had no significant effect on the basal level of IL-8 (Fig. 4A) or MCP-1 (Fig. 4B) protein expression in HUVECs.

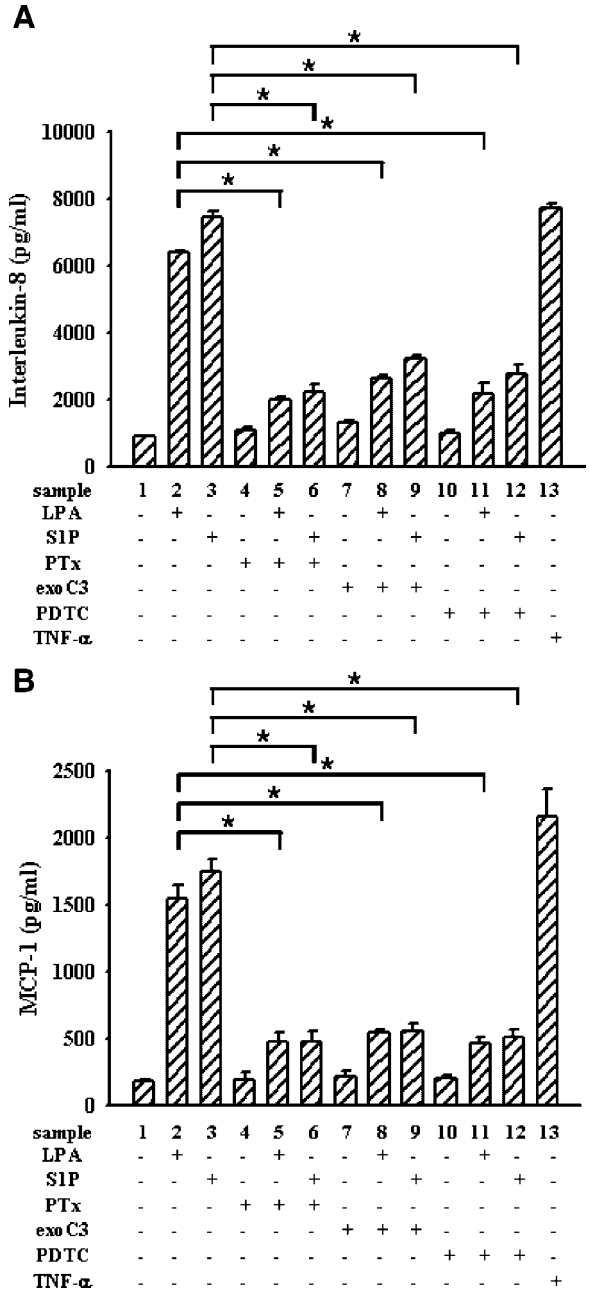

Fig. 4. LPA- and S1P-increased IL-8 and MCP-1 protein expressions are mediated through a Gi-, Rho-, and NF-κB-dependent mechanism. HUVECs were treated with control media alone, 10 ng/ml of PTx for 12 hr or 1 μg/ml of C3 exotoxin, or 200 μM of PDTC for 16 hr. Treated cells were then treated with 5 μM of LPA or S1P for 24 hr. IL-8 (**A**) and MCP-1 (**B**) protein expression levels were measured by an ELISA ($*P < 0.05$). All data are expressed as the mean ± SE. Similar experiments were repeated three times, and a representative result is shown in the figure.

However, the stimulatory effects of 5 μM of LPA or S1P on both IL-8 and MCP-1 protein expressions in HUVECs were totally suppressed by the toxin treatment (Fig. 4A,B). In addition, pretreatment with 1 μg/ ml of exotoxin C3, a specific inhibitor of Rho [Lee et al., 1999b, 2000] and 200 μM of PDTC, an inhibitor of the NFκB-dependent pathway [Da Silva et al., 2003], had no effects on basal IL-8 and MCP-1 protein expressions, but significantly inhibited LPL-enhanced IL-8 and MCP-1 protein expressions on HUVECs (Fig. 4A,B). These results suggest that the enhancement effects of LPL on IL-8 and MCP-1 protein expressions are $G_{i/o}$, Rho, and NFκB dependent. Similar results were also observed at the RNA level as detected by RT-PCR (Fig. 5).

LPA and S1P Upregulate Chemotactic Activity of LPL-Treated Conditioned Media of HUVECs

Since IL-8 and MCP-1 protein secretion in endothelial cells is responsible for enhancing the chemotactic activity toward monocyte recruitment, we tested the hypothesis that LPL treatments enhance the chemotactic activity of endothelial cells and subsequent monocyte recruitment. Conditioned media collected from 5 μM of LPA- or S1P-treated HUVECs enhanced cell migration of the human monocytic cell line, THP-1 (Fig. 6). Consistent with the RT-PCR and ELISA results, these enhancement effects were mediated through a Gi-, Rho-, and NF-κB-dependent mechanism (Fig. 6). However, LPA and S1P alone had no enhancement effects on THP-1 cell trans-well migration (Fig. 7). Furthermore, pre-incubation with 10 μg/ml functional blocking antibodies against human IL-8 and MCP-1 significantly inhibited the enhancement effect of LPL on THP-1 cell trans-well migration. On the contrary, normal mouse IgG (10 μg/ml) had no effects in these assays (Fig. 7A,B). These results imply that LPL treatment's enhancement of the chemotactic activity of endothelial cell is mediated through upregulation of the expressions of IL-8 and MCP-1.

IL-1 Mediates LPL-Induced IL-8 and MCP-1 mRNA Expressions

LPA and S1P enhanced IL-8 and MCP-1 mRNA expressions starting from 2 hr and peaking at 16 hr (Fig. 2). The early induction

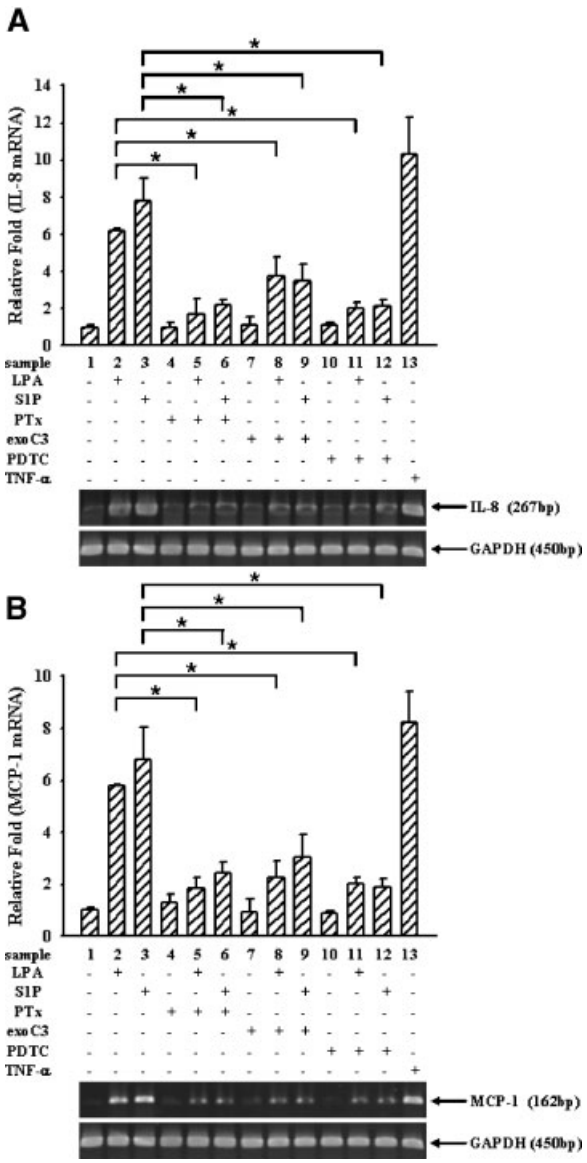

Fig. 5. Effects of a Gi inhibitor (PTX), Rho inhibitor (exoC3), and NF-κB inhibitor (PDTC) on LPL-stimulated IL-8 and MCP-1 mRNA expressions on HUVECs. HUVECs were pretreated with 10 ng/ml of PTx for 12 hr or 1 μg/ml of C3 exotoxin, or 200 μM of PDTC for 16 hr, followed by 5 μM of LPA or S1P treatment for an additional 16 hr. The IL-8 (**A**) and MCP-1 (**B**) mRNA expression levels were monitored by RT-PCR. Histograms represent quantification by PhosphorImager® of LPL-stimulated IL-8 and MCP-1 mRNA expression using ImageQuaNT® software (*$P < 0.05$). All data are relative multiples of expression compared to untreated cells. The data are representative of three experiments and are expressed as the mean ± SE.

of IL-8 and MCP-1 mRNAs by LPA and S1P may have been a direct consequence of the action of LPLs. Conversely, the apparent delay in induction (>12 hr) of MCP-1 and IL-8 mRNAs by LPLs suggests that these genes might be regulated by intermediary factors produced by endothelial cells in response to primary stimuli. A previous study indicated that the production of IL-8 and MCP-1 in endothelial cells was inhibited by functional blocking of the IL-1 receptor [Selvan et al., 1998]. Thus, we hypothesized that the induction of IL-8 and MCP-1 mRNAs by LPA and S1P might be regulated through IL-1. To test this hypothesis, we first determined the expression of IL-1β mRNA expression levels in LPL-treated HUVECs by RT-PCR. As shown in Figure 8A, IL-1β mRNA levels increased in both LPA- and S1P-treated samples in a time-dependent manner. In addition, pretreatment with 10 nM of AF12198, a low-molecular-weight antagonist which selectively binds the human type I interleukin (IL)-1 receptor [Akeson et al., 1996], had no effects on basal IL-8 or MCP-1 mRNA expression, but significantly inhibited LPA- and S1P-induced IL-8 and MCP-1 mRNA expression at time durations of 12∼24 hr. Moreover, pre-incubation with 10 μg/ml of IL-1β functional blocking antibody abolished most of the enhancement effects of LPLs on both IL-8 and MCP-1 mRNA expression at 12∼24 hr after treatment (Fig. 8B–E). These results suggest that the enhancement effects of LPLs on IL-8 and MCP-1 mRNA expressions are mediated through an IL-1-dependent mechanism.

DISCUSSION

The major findings of this study are that LPLs generated from activated platelets or other sources might enhance IL-8 and MCP-1 expressions by surrounding endothelial cells. The enhancement effects might be mediated through a $G_{i/o}$-, Rho-, and NFκB-dependent mechanism, which is consistent with the fact that Edg receptors activate these pathways. Furthermore, the time- and concentration-dependent activation of IL-8 and MCP-1 by LPLs is also consistent with a receptor-mediated mechanism. The enhancement effects of LPLs on IL-8 and MCP-1 expressions in human endothelial cells were principally responsible for monocyte recruitment. IL-1 expression might play critical roles in LPL-induced IL-8 and MCP-1 production.

Previous studies indicated that the chemokines IL-8 and MCP-1 are associated with several pathogenic processes underlying atherosclerosis [Liu et al., 1997; Nelken et al., 1991;

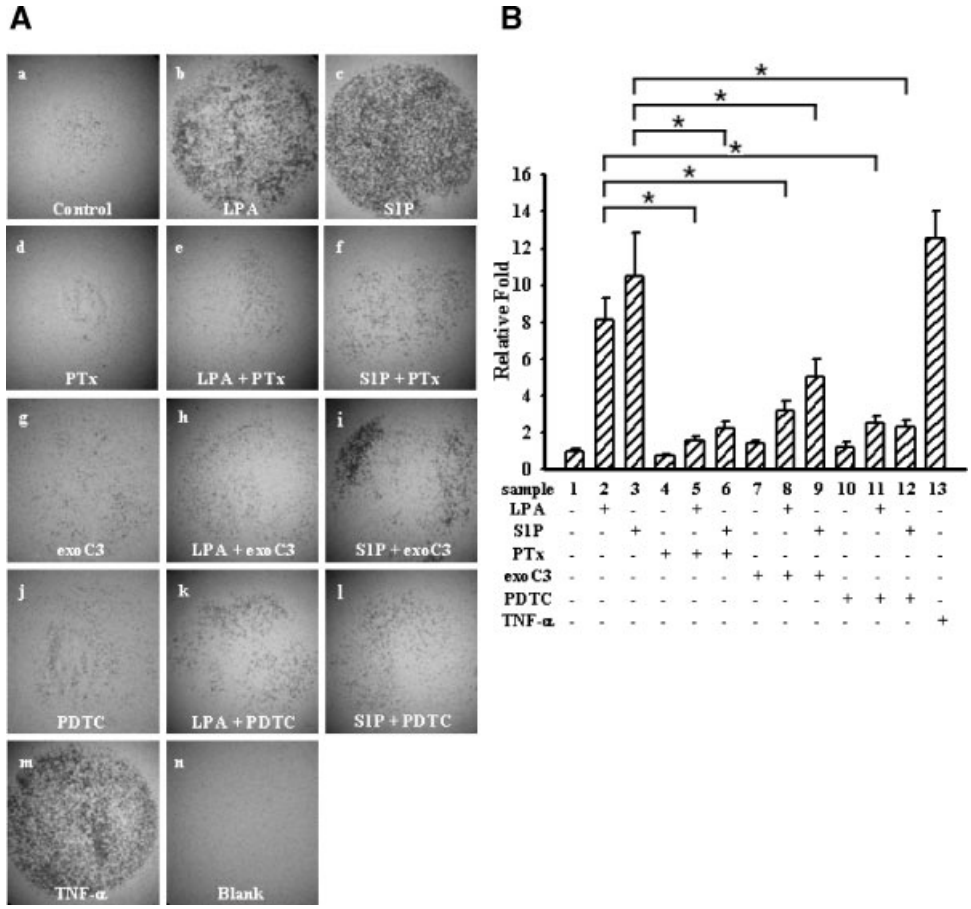

Fig. 6. LPA- and S1P-treated conditioned media of HUVECs upregulate the chemotactic activity towards THP-1 cells. **A**: HUVECs were pretreated with inhibitors as indicated for 16 hr, followed by 5 μM of LPA, or S1P treatment for 24 hr. The chemotactic activity was determined using a 48-well micro chemotaxis chamber. THP-1 cells were added to the upper chambers and allowed to migrate for 4 hr through an 8-μm porous membrane towards the lower chambers which contained either (**a**) untreated conditioned media, (**b**) LPA-treated conditioned media, (**c**) S1P-treated conditioned media, (**d**) PTx-treated conditioned media, (**e**) LPA + PTx-treated conditioned media, (**f**) S1P + PTx-treated conditioned media, (**g**) exoC3-treated conditioned media, (**h**) LPA + exoC3-treated conditioned media, (**i**) S1P + exoC3-treated conditioned media, (**j**) PDTC-treated conditioned media, (**k**) LPA + PDTC-treated conditioned media, (**l**) S1P + PDTC-treated conditioned media, (**m**) TNF-α-treated conditioned media, or (**n**) media alone which had been added as indicated. Cells which migrated to the lower chamber were fixed, stained, and photographed by a light microscope. **B**: Histograms represent quantification results of migrated THP-1 cells ($^*P < 0.05$). Migrated THP-1 cells on the membrane were stained with crystal violet and quantified by OD540. All data are relative multiples of expression compared to untreated cells. The data are representative of three experiments and are expressed as the mean ± SE.

Namiki et al., 2002; Wang et al., 2002]. Moreover, IL-8 and MCP-1 are expressed during phase-specific infiltration of subsets of leukocytes in human wound healing [Engelhardt et al., 1998]. In previous study, we showed that LPLs are wound-healing factors in the endothelium [Goetzl and An, 1998; Lee et al., 2000]. Another report also suggested that LPA facilitates wound healing in in vivo systems [Balazs et al., 2001]. Since IL-8 and MCP-1 are important regulators in leukocytes and endothelial cells that initiate leukocyte recruitment in blood vessels, this suggests that the facilitating effects of LPLs on wound healing might partially be mediated through enhancement of IL-8 and MCP-1 expressions. These results suggest that the effects of LPLs on leukocyte recruitment during wound healing occur at multiple levels. This is consistent with the fact that when a tissue is mechanically wounded, LPLs are released from activated platelets, which might act as one of the initiating signals for the subsequent wound-healing process. Our current study provides evidence that lysophospholipids (LPLs) might be vital mediators in the wound-healing process.

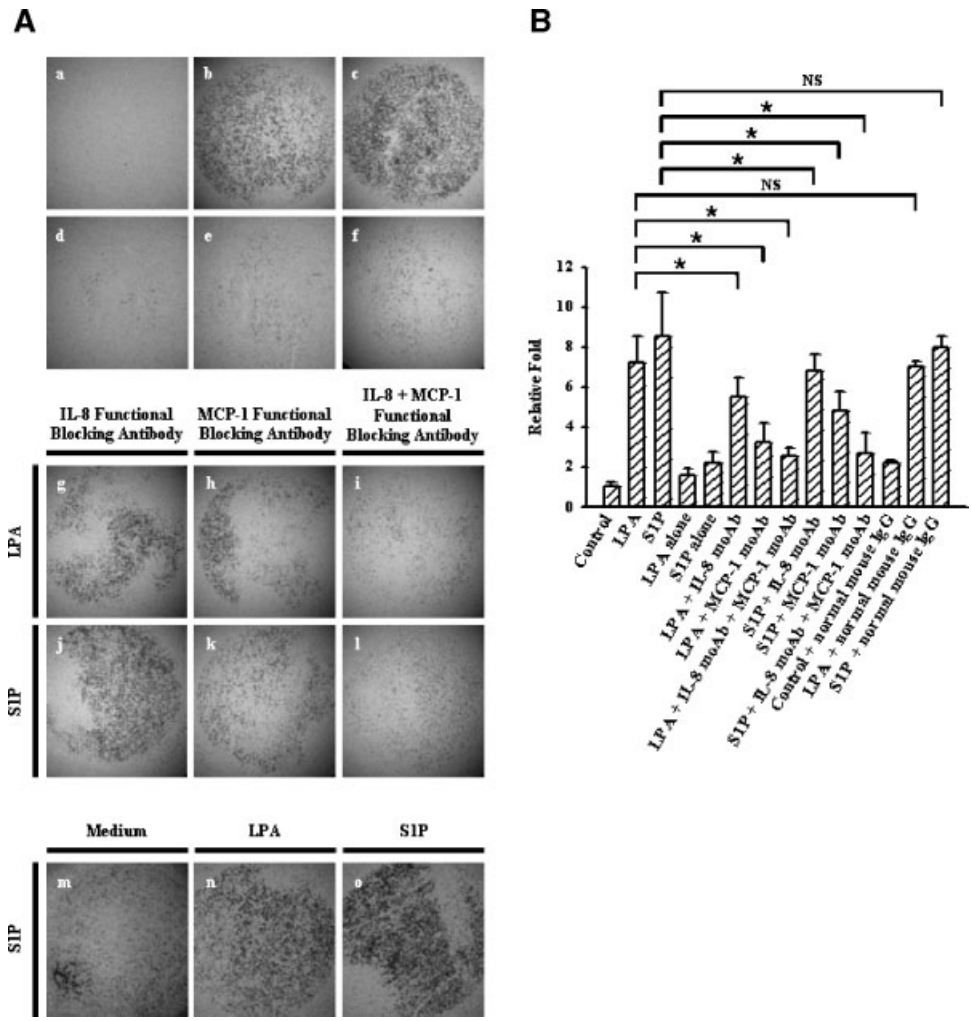

Fig. 7. Functional blocking antibodies for IL-8 and MCP-1 inhibit the chemotactic activity of conditioned media from LPL-treated HUVECs. The chemotactic activity was determined using a 48-well micro chemotaxis chamber as described in Figure 6. **A**: The lower chamber was filled with either (**a**) media alone, (**b**) LPA-treated conditioned media, (**c**) S1P-treated conditioned media, (**d**) untreated conditioned media, (**e**) media + LPA, (**f**) media + S1P, (**g**) LPA-treated conditioned media + IL-8 moAb, (**h**) LPA-treated conditioned media + MCP-1 moAb, (**i**) LPA-treated conditioned media + IL-8 moAb + MCP-1 moAb, (**j**) S1P-treated conditioned media + IL-8 moAb, (**k**) S1P-treated conditioned media + MCP-1 moAb, (**l**) S1P-treated conditioned media + IL-8 moAb + MCP-1 moAb, (**m**) media alone + normal mouse IgG, (**n**) LPA-treated + normal mouse IgG, or (**o**) S1P treated + normal mouse IgG as indicated. **B**: Histograms represent quantification of migrated THP-1 cells ($*P < 0.05$) as described in Figure 6. NS indicates that no significant difference was observed. All data are relative multiples of expression compared to untreated cells. The data are representative of three experiments and are expressed as the mean ± SE.

By RT-PCR, we found that LPA- and S1P-upregulated IL-8 and MCP-1 mRNA expressions peaked at 16 hr (Fig. 2). These results imply that the enhancement effect of LPLs on IL-8 and MCP-1 are mediated through a long-term activation mechanism which is prolonged to 16 hr. Selvan's group observed that a complement induced IL-8 and MCP-1 expressions in human endothelial cells through both IL-1-dependent and -independent mechanisms [Selvan et al., 1998]. Those results indicated that LPL-induced IL-8 and MCP-1 mRNA expressions also act in a biphasic manner. The latter phase of IL-8 and MCP-1 expressions might require the production of IL-1. IL-1 also acts as a mediator in regulating ICAM-1 expression in human endothelial cells [Gawaz et al., 2000]. These findings imply that LPL-enhanced IL-8 and MCP-1 gene expressions might require IL-1 as an intermediated mediator. By RT-PCR, IL-1 mRNA expression was found to be upregulated by both LPA and S1P. Moreover, the enhancement effects of LPLs on IL-8 and MCP-1 mRNA expressions at 12~24 hr

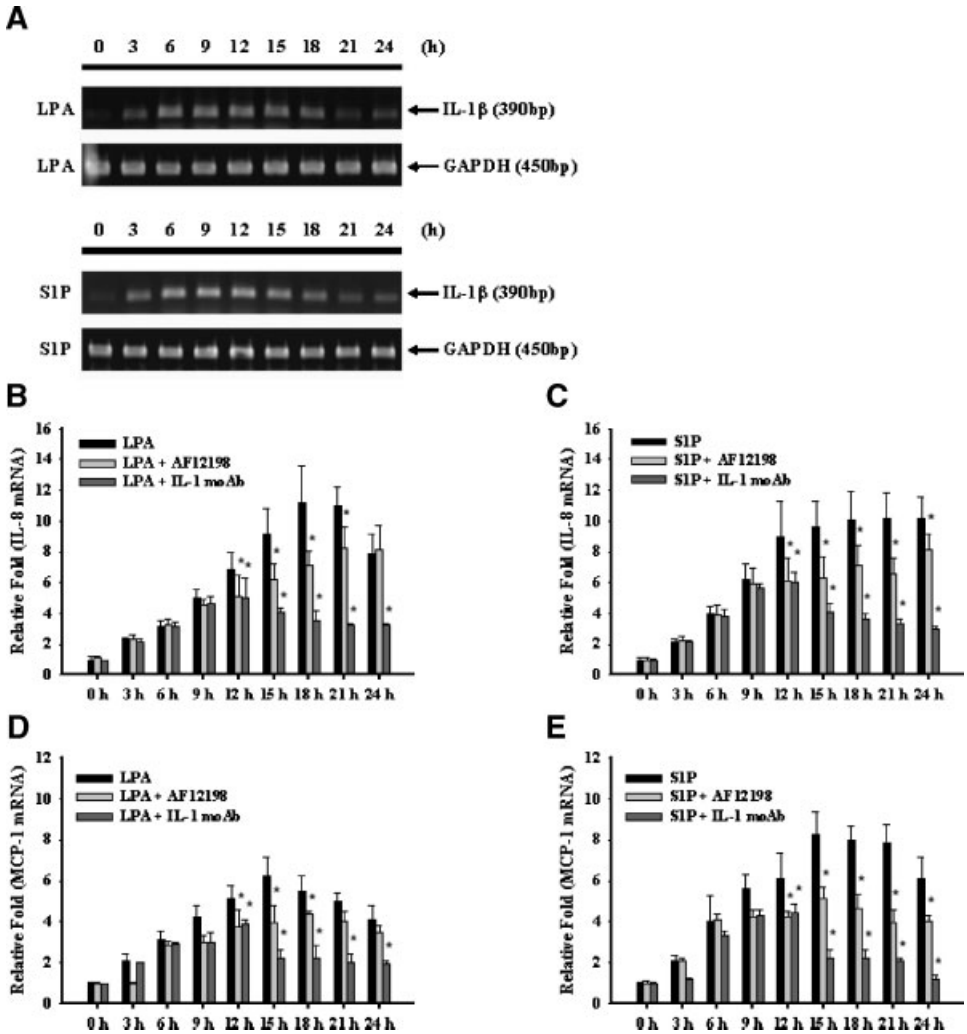

Fig. 8. LPA- and S1P-increased IL-8 and MCP-1 mRNA expressions are mediated through an IL-1-dependent mechanism. **A**: HUVECs were incubated with LPA (5 μM) or S1P (5 μM) for various times as indicated. RNAs from treated cells were harvested and subjected to RT-PCRs using specific primer sets for human IL-1β or GAPDH. Similar experiments were repeated three times, and a representative result is shown in the figure. Effects of AF12198 or IL-1 functional blocking antibody on IL-8 (**B** and **C**) and MCP-1 (**D** and **E**) mRNA expressions were evaluated after HUVECs were treated with 5 μM of LPA or S1P for various times as indicated (*$P < 0.05$). HUVECs were pre-incubated with control media alone (black bar), 10 nM of AF12198 (white bar) or 10 μg/ml of IL-1 functional blocking antibody (gray bar) for 16 hr. Treated cells were then treated with 5 μM of LPA or S1P for various times as indicated. The IL-8 and MCP-1 mRNA expression levels were assessed by RT-PCR as described above. Histograms represent quantification of RT-PCR corrected with GAPDH and analyzed by PhosphoreImager® using ImageQuaNT® software. All data are relative multiples of expression compared to untreated cells. *Significant differences between relative multiples in the absence and presence of AF12198 (*$P < 0.05$). The data are representative of three experiments and are expressed as the mean ± SE.

were blocked by pretreatment with AF12198, an IL-1 receptor antagonist or pre-incubation with IL-1β functional blocking antibody (Fig. 8). However, a significant induction of both cytokines at <12 hr is not affected by either IL-1 antagonist or antibody treatments. Therefore, it appears that there are at least two separate mechanisms involved in LPL-induced IL-8 and MCP-1 expression: an early direct LPL-dependent mechanism and a late IL-1-dependent mechanism. In addition, the enhancement effects of LPLs on IL-8 and MCP-1 mRNA, or protein expressions were blocked by inhibitors specific for $G_{i/o}$, Rho, and NF-κB at 16 hr (Figs. 4 and 5). It appears that these signaling pathways might be related to IL-1 induction and involved in the late IL-1-dependent mechanism. These results are consistent with our finding that LPL-induced IL-8 and MCP-1 mRNA expressions were prolonged to 16 hr due to

LPL-induced IL-1 production. It appears from these observations that IL-1 might be produced as an autocrine factor from endothelium that contributes to extend IL-8 and MCP-1 expression after 12 hr. The present study revealed that LPL might first induce IL-8 and MCP-1 expression directly. However, a late phase induction might require IL-1 as an intermediator.

IL-1 has been implicated in atherosclerosis in several reports [Hasdai et al., 1996; Ross, 1999], and it can be detected in atherosclerotic plaque [Wang et al., 1989], luminal endothelial cells, and macrophages [Tipping and Hancock, 1993]. IL-1 is expressed during monocyte–endothelial cell interactions, resulting in increased IL-8 and MCP-1 expressions [Fan et al., 1993; Lukacs et al., 1995]. In addition, IL-1 also participates in a self-augmentation induction mechanism, which allows a positive-feedback mechanism to amplify the effects of these cytokines within a local milieu [Dinarello, 1989]. Therefore, our results suggest that platelet-derived LPLs might facilitate wound-healing processes through complex cell–cell interactions in the local environment, which might contribute to the formation of atherosclerosis.

Recent studies reported that sphingosine kinase (SphK), the enzyme that catalyzes the formation of S1P from sphingosine, is involved TNF-α-induced MCP-1 expression in endothelial cells [Chen et al., 2004]. Exposure of A549 epithelial lung carcinoma cells to both TNF-α and IL-1β also resulted in a rapid activation of SphK1) and release of S1P from A549 cells [Billich et al., 2005]. These evidences indicated that LPL-induced IL-1β expression might contribute to SphK-mediated S1P release. These new generated S1P possibly results in MCP-1 expression indirectly in endothelial cells at late phase (>12 hr). In addition, it is conceivable that other pro-inflammatory cytokines such as TNF-α might participate in S1P-induced MCP-1 expression indirectly through activation of SphK in endothelial cells.

Several groups have reported the effects of LPA and S1P on neutrophil chemotaxis toward the endothelium. In one study, S1P inhibited transendothelial migration and invasiveness of neutrophils into HUVEC-covered collagen layers [Kawa et al., 1997]. The apparent diversity of the effects of LPA and S1P on leukocyte–endothelial cell interactions may be attributed to the use of different leukocytes and endothelial cell types. In our report, we clearly showed that both LPA and S1P are potent stimulators of HUVECs for enhancing the trans-well migration of THP-1 cells, and this is likely due to the enhancement of IL-8 and MCP-1 expressions in HUVECs. In addition, preincubation of functional blocking antibody against MCP-1 seemly showed stronger inhibitory effects than IL-8. These results indicated that LPLs upregulating chemotactic activity of endothelial cells on chemoattracting monocytes through enhancing MCP-1 expression in endothelial cells. Itagaki's group observed that LPA does not trigger PMN chemotaxis [Itagaki et al., 2005] consistent with our current observation that LPA alone had no enhancement effects on THP-1 cell trans-well migration. Our results suggest that platelet-derived LPLs enhance leukocyte recruitment of endothelial cells, consistent with the fact that they might be important regulators during wound healing and the formation of atherosclerosis.

Concentration of S1P in circulation plays different roles in regulating monocyte adhesion to endothelium. Previous studies [Xia et al., 1998; Lee et al., 2004] reported that S1P can induce VCAM-1, E-selectin, and ICAM-1 expression in endothelial cells contributed monocyte–endothelium adhesion. These investigators used high concentrations of S1P (5–20 μM). However, Bolick et al. [2005] reported that S1P at 0.1 μM concentrations can inhibit TNF-α-induced monocyte adhesion to endothelium. In high concentration range of S1P (>1 μM) activates NF-κB signaling pathway [Xia et al., 1998]. However, at low concentration of S1P (<0.5 μM) found no activation of NF-κB [Bolick et al., 2005]. Therefore, the different concentrations of S1P in circulation have different physiological functions in regulating monocyte–endothelium interactions through different signaling pathways.

Previous studies have reported that LPA and S1P induce IL-8 expression and secretion in human ovarian cancer cell [Schwartz et al., 2001] and human bronchial epithelial cells [Essler et al., 2000; Cummings et al., 2004]. In addition, these enhancement effects are NF-κB dependent [Cummings et al., 2004; Fang et al., 2004]. A recent study has demonstrated that the translocation of NF-κB is necessary for endothelial activation with the induction of *IL-8* and *MCP-1* genes [Kilgore et al., 1997]. Our current observation showed that LPA and S1P enhance IL-8 expression through a NF-κB-dependent

manner in HUVECs consistent with these previous studies. These results demonstrated that LPA and S1P might regulate various cellular biological functions in different cell types through enhancing NF-κB-mediated IL-8 expression.

Chemokines, which are responsible for the recruitment of leukocytes, play a crucial role as multifunctional mediators of inflammation by modulating key functions of immunocytes [Imhof and Dunon, 1995]. Therefore, delineating how LPLs affect the production of various chemokines at sites of inflammation may clarify the etiology of many diseases, including atherosclerosis. IL-8 and MCP-1 trigger firm adhesion of monocytes to the vascular endothelium under flowing conditions [Gerszten et al., 1999]. In our previous study, we confirmed that LPLs can enhance the firm adhesion of monocytes to endothelial cells through upregulation of ICAM-1 expression on endothelial cells [Lee et al., 2004]. These LPL-induced monocytes–endothelium interactions are considered to participate in wound healing and normal tissue remodeling in inflammation process, which might correlated to atherosclerosis [Lee et al., 2002].

Therefore, we propose that IL-8 and MCP-1 expressions in endothelial cells, enhanced by LPLs, are correlated to the firm adhesion of monocytes to endothelial cells. In the present study, we showed that LPA and S1P enhance IL-8 and MCP-1 secretion from endothelial cells. The enhancement effects might be mediated through a $G_{i/o}$-, Rho-, and NFκB-dependent mechanism, which is consistent with the fact that Edg receptors activate this pathway. Furthermore, the time- and concentration-dependent activation of IL-8 and MCP-1 by LPLs is also consistent with a receptor-mediated mechanism. Contrary to LPL stimulation of ICAM-1 expression being mediated through a Rho-independent mechanism [Lee et al., 2004], the enhancement effects of IL-8 and MCP-1 toward LPL treatment are mediated through a Rho-dependent mechanism. These results suggest that LPLs might regulate inflammation processes through complicated mechanisms.

In summary, our results clearly indicate that LPLs increase IL-8 and MCP-1 mRNA, and protein levels in time- and concentration-dependent manners. This induction is inhibited by specific inhibitors for $G_{i/o}$, Rho, and NF-κB. Furthermore, the enhancement effects of LPLs on IL-8 and MCP-1 expressions in HUVECs are responsible for the chemoattraction of leukocytes to the endothelium. Moreover, the mRNA and protein expressions of IL-8 and MCP-1 in response to LPLs are at least partially mediated through IL-1. Our results suggest that LPLs might be important physiological regulators of recruitment of leukocytes to the endothelium. Therefore, these lipids might play an important role in the regulation of wound healing and possibly the generation of atherosclerosis.

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