Role of ether-linked lysophosphatidic acids in ovarian cancer cells

Jun Lu,* Yi-jin Xiao,* Linnea M. Baudhuin,*,§ Guiying Hong,* and Yan Xu[1],*,†,§

Department of Cancer Biology,* Lerner Research Institute, and Department of Gynecology and Obstetrics,† Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195; and Department of Chemistry,§ Cleveland State University, 24th and Euclid Avenue, Cleveland, OH 44115

Abstract Naturally occurring alkyl- and alkenyl-lysophosphatidic acids (al-LPAs) are detected and elevated in ovarian cancer ascites compared with ascites from non-malignant diseases. Here we describe the biological functions and signaling properties of these ether-linked LPAs in ovarian cancer cells. They are elevated and stable in ovarian cancer ascites, which represents an in vivo environment for ovarian cancer cells. They stimulated DNA synthesis and proliferation of ovarian cancer cells. In addition, they induced cell migration and the secretion of a pro-angiogenic factor, interleukin-8 (IL-8), in ovarian cancer cells. The latter two processes are potentially related to tumor metastasis and angiogenesis, respectively. Al-LPAs induced diverse signaling pathways in ovarian cancer cells. Their mitogenic activity depended on the activation of the $G_{i/o}$ protein, phosphatidylinositol-3 kinase (PI3K), and mitogen-activated protein (MAP) kinase kinase (MEK), but not p38 mitogen activated protein kinase (MAP kinase). S473 phosphorylation of protein kinase B (Akt) by these lipids required activation of the $G_{i/o}$ protein, PI3K, MEK, p38 MAP kinase, and Rho. However, T308 phosphorylation of Akt stimulated by al-LPAs did not require activation of p38 MAP kinase. On the other hand, cell migration induced by al-LPAs depended on activities of the $G_{i/o}$ protein, PI3K, and Rho, but not MEK. These data suggest that ether-linked LPAs may play an important role in ovarian cancer development.—Lu, J., Y-j. Xiao, L. M. Baudhuin, G. Hong, and Y. Xu. **Role of ether-linked lysophosphatidic acids in ovarian cancer cells. *J. Lipid Res.* 2002.** 43: **463–476.**

Supplementary key words protein kinase B • mitogen activated protein kinase • alkyl-lysophosphatidic acid • alkenyl-LPA • ovarian cancer

Lysophosphatidic acid (LPA) is a bioactive lysolipid that is involved in a broad range of biological processes in a variety of cellular systems (1, 2). LPA induces cell proliferation or differentiation, prevents apoptosis induced by stress or stimuli, induces platelet aggregation and smooth muscle contraction, and stimulates cell morphologic changes, cell adhesion, and cell migration (1–5). LPA has been shown to be involved in angiogenesis, wound healing, and inflammatory processes (6–15). LPA exerts many of its effects by binding to G protein-coupled receptors (GPCRs), resulting in a cascade of intracellular signaling activations (2, 16). Three endothelial differentiations genes [endothelial differentiations gene (Edg) 2, 4, and 7] have been identified as receptors for LPA (7, 17–19). LPA stimulates G_i-mediated extracellular mitogen-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) activation, G_q-mediated phospholipase C (PLC) and protein kinase C (PKC) activation, and $G_{12/13}$-mediated Rho activation (1, 2).

We have previously identified a growth-stimulating factor, ovarian cancer activating factor (OCAF), in ascites from patients with ovarian cancer. OCAF is composed of various species of LPAs (with different fatty acid side chains) (20). OCAF and synthetic 18:1-acyl-LPA stimulate growth of ovarian, breast, and Jurkat cells (21, 22). Acyl-LPA (18:1) also regulates other cellular activities. It enhances cell adhesion/attachment (23), stimulates interleukin-8 (IL-8) production from ovarian cancer cells (24), and synergizes with other agents, such as thrombin agonists, noradrenaline, ADP, and arachidonic acid, to induce strong platelet aggregation (5). LPA has been shown to decrease *cis*-diamminedichloroplatinum-induced cell death (25), prevent cell apoptosis (26), and induce urokinase secretion (27) and vascular endothelial growth factor expression in human ovarian cancer cells (15). In addition, we have shown that acyl-LPAs are elevated in plasma from patients with ovarian cancer and may represent a useful marker for the early detection of ovarian cancer (28).

Abbreviations: Akt, protein kinase B; al-LPAs, alkyl- and alkenyl-lysophosphatidic acids; Edg, endothelial differentiations gene; ECL, enhanced chemiluminescence; ERK, extracellular mitogen-regulated kinase; ESI-MS, electrospray ionization mass spectrometry; GPCR, G protein-coupled receptor; IL-8, interleukin-8; LPA, lysophosphatidic acid; MAP kinase, mitogen activated protein kinase; MEK or MKK, MAP kinase kinase; MS-MS, tandem mass spectrometry; MRM, multiple reaction monitoring; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OCAF, ovarian cancer activating factor; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; PTX, pertussis toxin; S1P, sphingosine-1-phosphate.

[1] To whom correspondence should be addressed at the Department of Cancer Biology, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195.
e-mail: xuy@ccf.org

There are three subclasses of LPA: acyl-, alkyl-, and alkenyl-LPAs. The latter two subclasses of LPAs (al-LPAs) differ from acyl-LPA in that the fatty acid chain is linked to the glycerol backbone through an ether or a vinyl, rather than an ester bond. The majority of research work on LPA has been performed on acyl-LPAs (19, 29), although the effect of synthetic alkyl-LPA on platelet aggregation was reported decades ago (30). Most alkyl-LPA work was performed using synthetic alkyl-LPA (30, 31), and the naturally occurring al-LPAs have been reported only in recent years (12, 32–34).

We have recently developed an electrospray ionization mass spectrometry (ESI-MS)-based method to analyze lysolipids in body fluids (35) and found that, in addition to acyl-LPAs, ascites from patients with ovarian cancer contain elevated al-LPAs (including 16:0-/18:0-alkyl-LPA and 16:0-/18:0-alkenyl-LPA), when compared with ascites from patients with benign diseases and endometrial cancer (35). These results implicate that al-LPAs may have potential pathophysiological roles in ovarian cancer.

In the present study, we describe that al-LPAs were more stable than acyl-LPAs in ascites. These lipids stimulated DNA synthesis and proliferation of ovarian cancer cells through G_i-, PI3K-, and mitogen activated protein (MAP) kinase kinase (MEK)-dependent pathways. Al-LPAs and acyl-LPAs induced migration of ovarian cancer cells through collagen I-coated membranes and this activity required the activation of G_i, and was partially dependent on PI3K activity. In addition, al-LPAs stimulated IL-8 production. Similar to acyl-LPAs as we observed recently (unpublished observations), al-LPAs activated Akt kinase and induced a Rho-, PI3K-, and MEK-dependent S473 phosphorylation of Akt.

MATERIALS AND METHODS

Chemicals

LPAs (16:0, 18:0, and 18:1), lyso-platelet activating factor (lyso-PAF), and other lysophospholipids were purchased from Avanti Polar Lipids (Birmingham, AL). Lyso-plasmalogen phosphatidylethanolamine (alkenyl-LPE) was obtained from Matreya, Inc. (Pleasant Cap, PA). LY294002, PD98059, and SB203580 were obtained from Biomol (Plymouth Meeting, PA). Wortmannin was obtained from Sigma (St. Louis, MO). Pertussis toxin (PTX) was purchased from Life Technologies, Inc. (Rockville, MD). Precoated silica gel 60 TLC plates were obtained from EM Science (Gibbstown, NJ). HPLC grade methanol (MeOH), chloroform, ammonium hydroxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and hydrochloric acid (HCl) were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). [^{3}H]thymidine was from NEN Life Science Products, Inc. (Boston, MA). Anti-phospho-S473-Akt, anti-phospho-T308-Akt, anti-phospho-ERK, and anti-ERK were obtained from Cell Signaling Technology (Beverly, MA). Anti-MEK2 and anti-p38 were from StressGen (Victoria, BC, Canada).

Cell lines and cell culture

HEY and SKOV3 cells were from Dr. G. Mills (The University of Texas M. D. Anderson Cancer Center, Houston, TX) and American Type Culture Collection (Manassas, VA), respectively, and maintained in RPMI 1640 medium containing 10% FBS at 37°C with 5% CO_2. All cells were cultured in serum-free media for 18–24 h prior to lipid treatment except in the cell migration experiments. For transient transfections, cells were plated into 35 mm dishes and transfected with DNA using LipofectAMINE (Life Technologies, Inc.) and Transfection Booster Reagents (Gene Therapy Systems, San Diego, CA) according to the manufacturers' instructions. Dominant negative MEK was from Dr. D. Templeton, Case Western Reserve University. Kinase inactive p38 was from Dr. Bryan R.G. Williams, Cleveland Clinic Foundation. The C3-exoenzyme construct was provided by Dr. Alan Wolfman, Cleveland Clinic Foundation. Dominant negative Akt was from Dr. Kumliang Guom, University of Michigan.

Nonradioactive immunoprecipitation Akt kinase assay

The Akt kinase assay was performed with the Nonradioactive Akt Kinase Assay Kit (Cell Signaling Technology, Beverly, MA) according to the manufacturer's instructions. All reagents were provided with the kit. Briefly, cells were treated with al-LPAs, rinsed with ice-cold phosphate-buffered saline, and then lysed in cell lysis buffer. Immunoprecipitation was carried out using immobilized Akt 1G1 monoclonal antibody. The immunoprecipitate was then incubated with GSK-3 fusion protein and ATP in kinase buffer. Western analysis was used to determine the extent of GSK-3 phosphorylation by active Akt using a phospho-GSK-3 α/β (Ser21/9) antibody.

Extraction and quantitation of alkyl- and alkenyl-LPAs from ascites

Extraction of alkyl- and alkenyl-LPAs from ascites was performed as described previously (35, 36). The stability of different LPA species was tested. Briefly, ascites samples were stored at 4°C for different time periods and lipids in ascites were extracted with chloroform and methanol in the presence of HCl. The chloroform phase was dried and lipids were separated on TLC plates. Different LPA species were eluted from TLC plates with a mixture of methanol and chloroform (2:1, v/v). ESI-MS and tandem mass spectrometry (MS-MS) analyses for the quantitation of alkyl- and alkenyl-LPAs were performed using a Micromass Quattro II Triple Quadrupole Mass Spectrometer. All quantitative analyses were performed in the multiple reaction monitoring (MRM) mode as described previously (35).

Preparation of alkyl- and alkenyl-LPAs

Alkyl- and alkenyl-LPAs were prepared through hydrolysis of the corresponding lyso-PAF or lyso-plasmalogen phosphatidylethanolamine (alkenyl-LPE), respectively, by phospholipase D (PLD) (Calbiochem, La Jolla CA). Briefly, 1 mg of alkenyl-LPE or lyso-PAF was dispersed in 0.1 ml of 0.04 M Tris buffer, pH 8.0, containing 0.05 M $CaCl_2$ and 1% Triton-X100. After addition of the enzyme (four units of PLD in 15 μl of 0.01 M Tris-HCl, pH 8.0), the sample was mixed vigorously. The reaction vessel was sealed tightly and the contents were rotated overnight at room temperature. After the incubation period, the mixture was extracted with 1.2 ml mixture of chloroform-MeOH-HCl (5:4:0.2, v/v/v). The chloroform layer was evaporated under a stream of nitrogen and the residue was dissolved in 50 μl chloroform-MeOH (1:2, v/v). The substrate and the product were separated on a TLC plate using a solvent system of chloroform-MeOH-NH_4OH (65:35:5.5, v/v/v) and the product was eluted from the plate by extracting with 2 ml of chloroform-methanol (1:2, v/v) twice and then dried under N_2. The lipid product was identified and quantified by ESI-MS and then dissolved in methanol to make a 1 mM solution.

DNA synthesis and MTT assays

HEY cells were plated in 96-well plates, serum-starved for 16–24 h, and treated for 24 h with different concentrations of al-LPAs

in F12-DMEM (1:1, v/v) medium supplemented with 0.1% fatty acid-free BSA, insulin, transferrin, and selenium. For the DNA synthesis assays, the cells were incubated with 0.15 μCi/well [^{3}H]thymidine for the last 18 h. Cells were harvested onto filter papers presoaked in 1% polyethyleneimine using an automated cell harvester, HARVEST 96 (Perkin-Elmer-Wallac, Inc.). Incorporated [^{3}H]thymidine was counted in a 1450 Microbeta Trilux Liquid Scintillation and Luminescence Counter (Perkin-Elmer-Wallac, Inc.). For cell proliferation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assays were used. Twenty μl of MTT solution (5 mg/ml) was added to each well and incubated at 37°C for the last 6 h of lipid treatment. The reduced MTT crystals were dissolved in 100 μl/well of a mixture of DMSO and 95% ethanol (1:1, v/v). The color developed was read by a plate reader (SpectraMax 340, Molecular Devices Corp, Sunnyvale, CA) at 595–655 nm.

Western blotting

HEY cells were plated in 6-well plates in RPMI 1640 with 10% FBS, serum-starved overnight, and then treated with or without al-LPAs in serum-free media for the indicated times. Cells were lysed on ice with Laemmli buffer containing 5% mercaptoethanol. The lysates were separated with 12% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Antibodies against S473 phosphorylated Akt, T308 phosphorylated Akt, or phosphorylated ERK1/2 were used to probe the membrane and the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech Inc., Piscataway, NJ) was used for detection. To normalize the amounts of protein loaded in each lane, the membranes were stripped and reprobed with antibodies against total Akt or ERK.

Cell migration assays

Chemotaxis was performed in a mini-Boyden chamber (Neuro Probe, Inc., Cabin John, MD) using Nucleopore polycarbonate filters (8 μm pore size) coated with a type-I collagen solution (100 μg/ml) (Vitrogen100, Collagen Corporation, Fremont, CA). Different concentrations of LPAs were added to the lower chamber. Checkerboard assays were performed as described by Okamoto et al. (37). HEY cells were starved for 3 h, trypsinized and resuspended at a concentration of 2.5×10^5 cells/ml in serum-free medium. The cell suspension (50 μl) was then placed in the upper chamber. After 4 h at 37°C, the cells that attached to the filters were fixed in 100% methanol and stained with Hematoxylin solution (Richard-Allan Scientific, Kalamazoo, MI). Cells that migrated to the lower phase of the membrane were counted under the microscope.

IL-8 ELISA assays

Cells were grown in 96-well plates, starved overnight, and treated with lipids for 6 h. The supernatants were collected and stored at −80°C. The IL-8 concentration was measured using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol with minor modifications as described previously (24). All analyses were carried out in triplicate. Optical densities were determined using a SpectraMax 340 plate reader (Molecular Devices, Sunnyvale, CA) at 650–490 nm.

RESULTS

Al-LPAs were more stable than acyl-LPAs in ascites from patients with ovarian cancer

We have previously compared the lysolipid content in 15 pairs of ascites samples from patients with ovarian cancer and non-malignant diseases, and reported that al-LPAs were elevated in ovarian cancer ascites (35). Four al-LPA species were detected in ascites samples: 16:0- and 18:0-alkyl LPAs, and 16:0- and 18:0-alkenyl-LPAs. The distribution of different al-LPA and acyl-LPA species in 15 ovarian cancer and 15 non-malignant ascites samples is shown in **Table 1**. al-LPA species in ascites account for approximately 12% of total LPAs (both al- and acyl-LPAs). We observed that al-LPAs were more stable in ascites stored at 4°C under sterile conditions (**Fig. 1A**). The average half-lives of acyl-LPAs and al-LPAs in ascites stored at 4°C were approximately 12 months and more than 2 years, respectively (results obtained from five ascites samples) (Fig. 1B).

Al-LPAs stimulated DNA synthesis and growth of HEY ovarian cancer cells

To determine the potential pathophysiological role of al-LPAs in ovarian cancer cells, we first examined the effects of al-LPAs on DNA synthesis and proliferation in HEY ovarian cancer cells. Alkyl-LPA (16:0) and 16:0-/18:0-alkenyl-LPA (the ratio of 16:0 to 18:0 was approximately 1:1) were synthesized as described in Materials and Methods. **Figure 2** shows the spectra of synthesized 16:0-/18:0-alkenyl- and 16:0-alkyl-LPAs. Each preparation contained a small amount of impurities, which were mainly derived from 16:0- and

TABLE 1. Statistical analysis of LPAs in 15 pairs of ascites samples from patients with ovarian cancer or non-malignant diseases[a]

	Alkyl-LPA		Alkenyl-LPA		Total al-LPAs	Acyl-LPA				Total acyl-LPAs	Total LPAs
	16:0	18:0	16:0	18:0		16:0	18:2	18:1	18:0		
	μM		*μM*		*μM*	*μM*				*μM*	*μM*
Ovarian cancer											
Minimum	0.3540	0.1046	0.0943	0.4688	1.0217	0.1498	0.0780	0.0017	0.0048	1.1002	2.6949
Maximum	3.8929	1.2483	0.6663	2.3318	7.1772	37.1935	3.7894	8.0704	11.1937	54.3211	59.6573
Mean	1.4800	0.6371	0.2906	1.3036	3.7113	11.2207	1.1806	2.4424	4.0650	18.9087	22.6134
Median	1.2620	0.6577	0.2651	1.2278	3.6384	12.1470	0.4485	1.8458	1.7882	19.4320	22.5316
Benign disease											
Minimum	0.0000	0.0000	0.0000	0.0164	0.0423	0.2779	0.0000	0.0392	0.0412	0.3966	0.4389
Maximum	0.4450	0.2541	0.0585	0.4081	1.1072	5.1070	0.9057	0.9932	0.6924	6.9865	7.5109
Mean	0.1430	0.0997	0.0082	0.1072	0.3580	1.8723	0.3325	0.3986	0.2582	2.8615	3.1956
Median	0.0928	0.0820	0.0000	0.0602	0.2287	1.3920	0.3129	0.3707	0.2199	2.3900	2.5705

[a] Sample collection, lipid extraction, and analyses were performed as described previously (35).

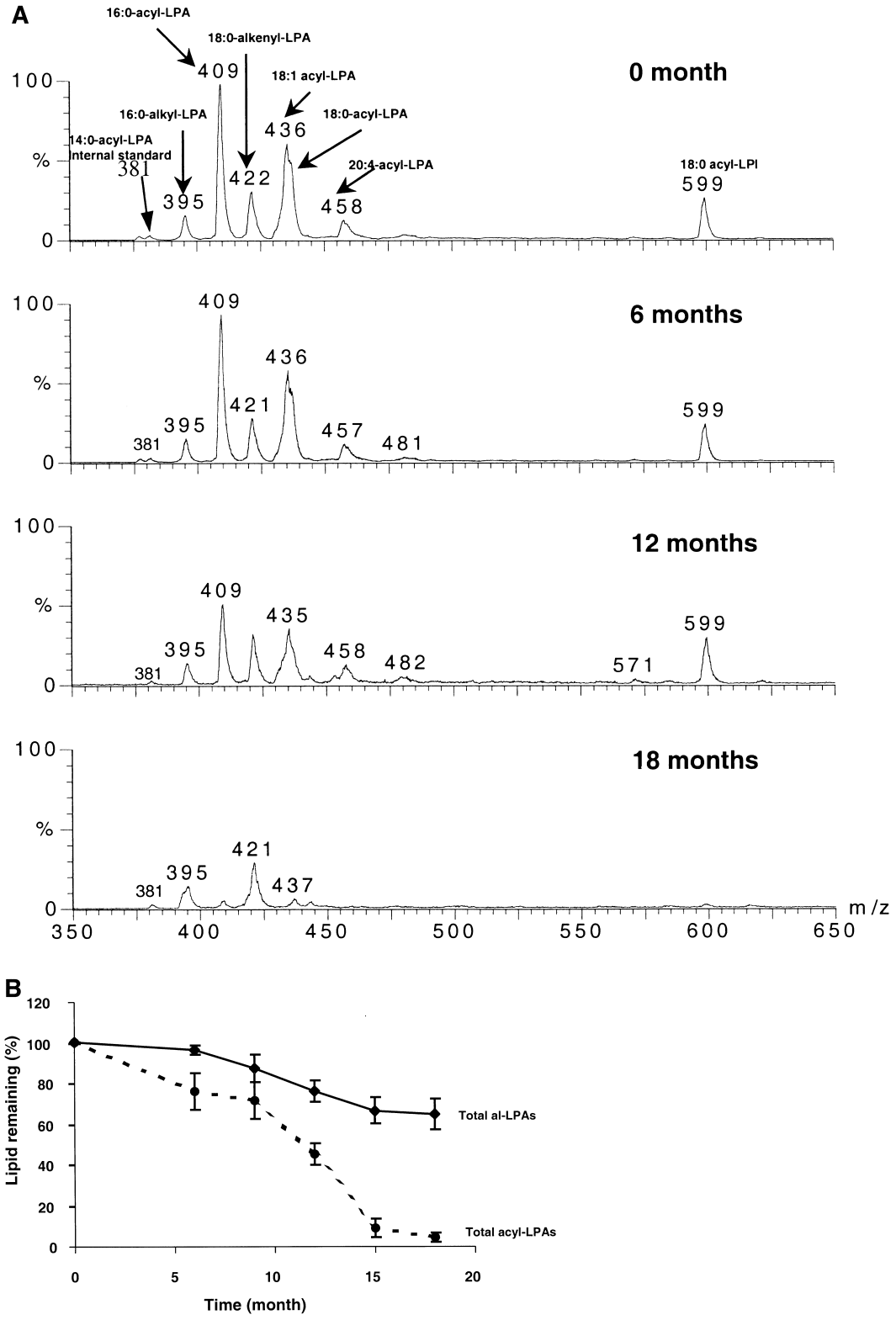

Fig. 1. Alkyl- and alkenyl-lysophosphatidic acids (al-LPAs) in ascites from ovarian cancer patients are more stable than acyl-LPAs. Al-LPAs and acyl-LPAs from ascites samples were extracted and analyzed as described in Materials and Methods. Five ascites samples from patients with ovarian cancer were stored at 4°C under sterile conditions. LPAs from 0.5 ml of ascites were analyzed at the time intervals as indicated. A: Electrospray ionization mass spectrometry (ESI-MS) spectra of LPAs from representative ovarian cancer ascites samples analyzed at 0, 6, 12, and 18 months. B: The stability of LPAs in five ovarian cancer ascites samples.

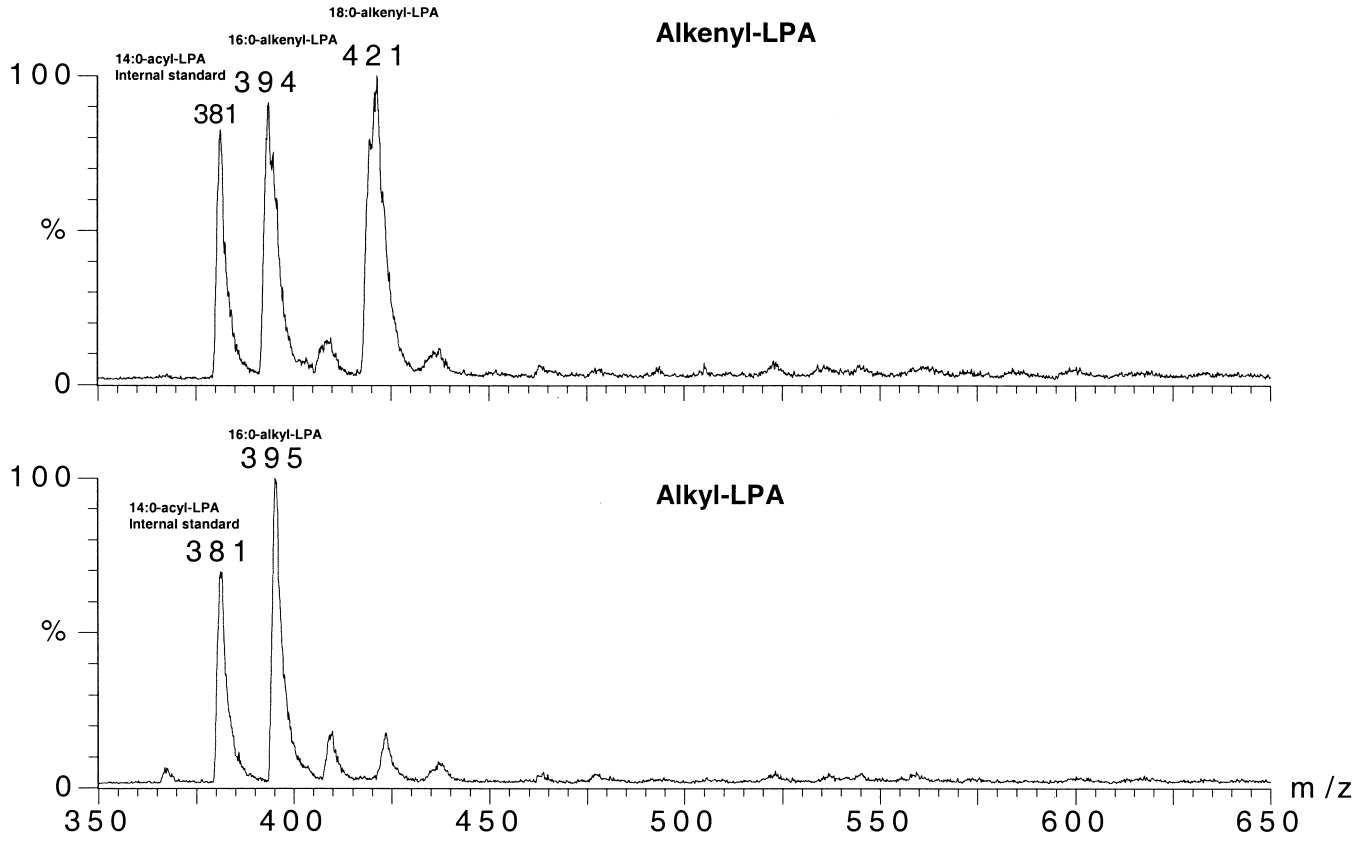

Fig. 2. The spectra of synthetic alkyl- and alkenyl-LPAs. al-LPAs were synthesized and analyzed as described in Materials and Methods. Al-LPAs were resuspended in methanol and 20 μl of al-LPAs containing 50 pmol of 14:0-acyl-LPA (internal standard) was used for MS analyses.

18:0-acyl-LPAs. The relatively low amount (<10% and <5% in the alkyl-LPA and the alkenyl-LPA preparations, respectively) of these impurities did not affect the activities tested in this study. Starved HEY cells were incubated with different concentrations of lipids (0.1–5.0 μM within the concentration range detected in ascites from patients with ovarian cancer) for 24 h. The effects of lipids on DNA synthesis were assessed by addition of [^{3}H]thymidine (0.15 μCi/well) and the effect of lipids on cell proliferation was measured by MTT dye reduction. Physiological concentrations of 16:0-alkyl-LPA (1–5 μM) and 16:0-/18:0-alkenyl-LPA (1–5 μM) increased [^{3}H]thymidine incorporation and MTT dye reduction to approximately 2-fold (**Fig. 3A** and **B**).

Al-LPAs activated ERK and Akt

We have shown in our recent studies that acyl-LPA induces ERK, p38, and Akt activation in HEY cells (unpublished observations). We sought to examine the activation of ERK and Akt induced by al-LPAs. Both alkyl- and alkenyl-LPAs activated Akt as assessed by an Akt kinase assay (**Fig. 4A**). West-

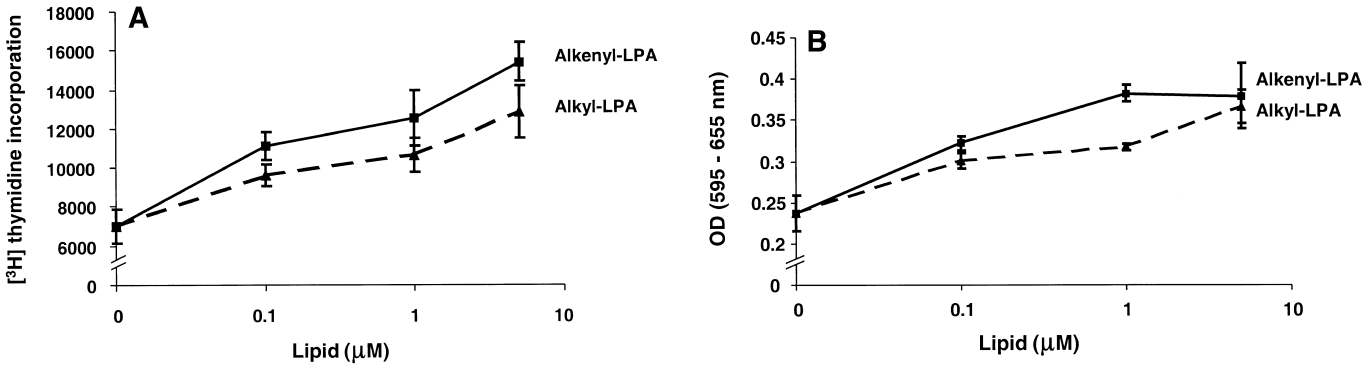

Fig. 3. Al-LPAs stimulated DNA synthesis in HEY cells. A: DNA synthesis was measured by using [^{3}H]thymidine incorporation as described in Materials and Methods. Starved cells were treated with al-LPAs (1–5 μM) for 24 h. B: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction was used to measure cell proliferation. Starved cells were treated with al-LPAs (1–5 μM) for 24 h. MTT solution was added and incubated at 37°C for the last 6 h of lipid treatment.

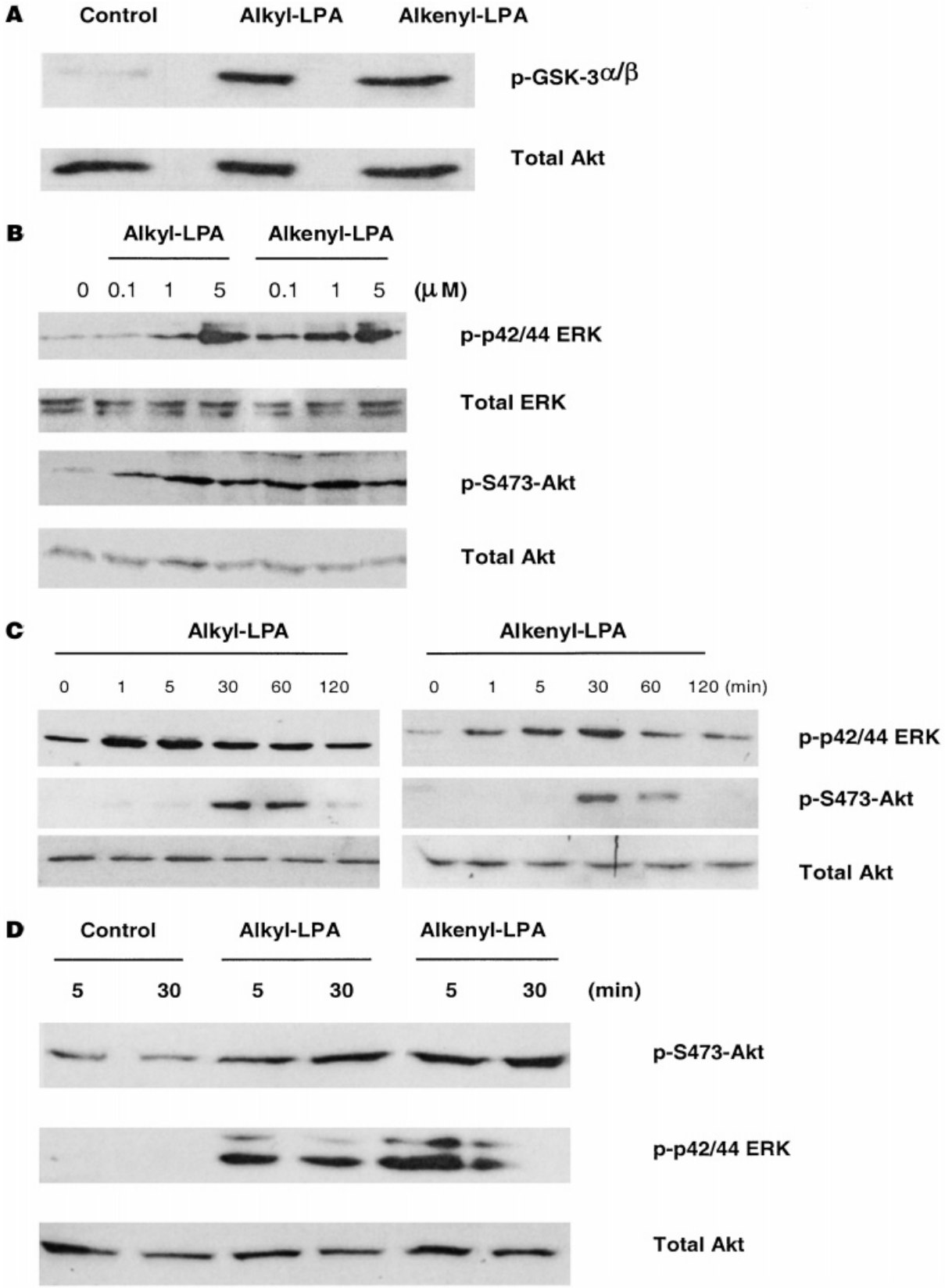

Fig. 4. Al-LPAs activated extracellular mitogen-regulated kinase (ERK) mitogen activated protein kinase (MAP kinase) and protein kinase B (Akt) in HEY and SKOV3 ovarian cancer cells. A: The kinase activity of Akt was performed with the Nonradioactive Akt Kinase Assay Kit according to the manufacturer's instructions. Starved HEY cells were treated with 2 μM al-LPAs for 30 min. B: Concentration-dependent Akt (30 min) and ERK (5 min) phosphorylation by al-LPAs. HEY cells were serum-starved for 18–24 h before stimulation with lipids. C: The time courses of ERK and Akt phosphorylation stimulated by alkyl-LPA (2 μM) or alkenyl-LPA (2 μM) for the indicated times in HEY cells. D: ERK and Akt phosphorylation induced by al-LPAs in SKOV3 cells. Starved SKOV3 cells were treated with 2 μM al-LPAs for the indicated times.

ern blot analyses of phosphorylated ERK and Akt (S473) were performed after HEY cells were treated with alkyl- or alkenyl-LPAs. Both alkyl- and alkenyl-LPAs (2 μM) induced a concentration- and time-dependent activation of ERK and a transient increase in the S473 phosphorylation of Akt (Fig. 4B and C). The optimal concentrations were 5 μM and 1 μM for al-LPAs to activate ERK and Akt, respectively (Fig. 4B). Concentrations higher than 5 μM were not tested, since they are out of the physiological concentration ranges of al-LPAs detected in ovarian cancer ascites (Table 1). The optimal times for induction of ERK and Akt phosphorylation by alkyl-LPA were 1–5 min and 30 min, respectively. Alkenyl-LPA induced maximal phosphorylation of both ERK and Akt at 30 min (Fig. 4C). Similarly, al-LPAs induced ERK and Akt phosphorylation in another ovarian cancer cell line, SKOV3 (Fig. 4D).

Pertussis toxin (PTX, a $G_{i/o}$ inhibitor; 100 ng/ml) partially, and two specific inhibitors of PI3K, LY294002 (10 μM) and wortmannin (100 nM), completely inhibited the activation of ERK and Akt induced by al-LPAs, suggesting that a PTX-sensitive G protein and PI3K are involved in phosphorylation of ERK and Akt (**Fig. 5A** and **B**).

Acyl-LPA-induced Akt activation is dependent on the activities of both MEK and p38, which is both cell line and stimulus specific (unpublished observations). In addition to our work, this MEK-dependent Akt activation/phosphorylation has been shown very recently in ultraviolet B- and serotonin-induced Akt activation (38, 39). To investigate whether al-LPAs activated the same signaling pathways as acyl-LPAs in HEY cells, we tested the effects of a panel of pharmacological and genetic inhibitors on the Akt phosphorylation induced by al-LPAs. Similar to acyl-LPAs, Akt phosphorylation at S473 was sensitive to both PD98059 and SB203580 (the specific inhibitors for MEK1/2 and p38, respectively) (Fig. 5C), suggesting that MEK, and potentially its downstream effector ERK, and p38 were required for Akt phosphorylation at S473 by al-LPAs. This was further confirmed by transfecting HEY cells with dominant negative forms of MEK and p38 (MEK/2A and p38/AGF) (Fig. 5D). We have developed an efficient transfection method in HEY cells (unpublished observations). Using both LipofectAMINE and Transfection Booster Reagents #3 (from Gene Therapy System, Inc., San Diego, CA), the transfection efficiency was increased from 15 ± 4% to 77 ± 6% (unpublished observations). Both these dominant negative forms of MAP kinases blocked Akt activation induced by al-LPAs, indicating that both MEK and p38 activities are required for al-LPA-induced S473 phosphorylation of Akt in HEY cells.

Since phosphorylation of both T308 and S473 are necessary for the full activation of Akt, we examined the ability of al-LPA to stimulate Akt T308 phosphorylation. Both alkyl- and alkenyl-LPAs (2 μM) were able to induce an approximately 3-fold increase in Akt phosphorylation at T308 in HEY cells (Fig. 5E). To determine whether phosphorylation at T308 required p38 MAP kinase, we pretreated cells with SB 203580 (0.5, 2.5, and 10 μM) followed by treatment with al-LPAs (Fig. 5E). Our results show that 0.5, 2.5, and 10 μM of SB203580 inhibited approximately 20%, 80%, and 100% phosphorylation at S473, respectively. However, even at 10 μM, the phosphorylation of T308 was not affected by SB 203580, suggesting that p38 MAP kinase was not required for this phosphorylation.

We have shown that acyl-LPA, but not a structurally similar lipid, sphingosine-1-phosphate (S1P), induces Akt phosphorylation via a Rho-dependent pathway (unpublished observations). We tested whether al-LPAs also require Rho for induction of S473 phosphorylation of Akt. Transient transfection of C3-exoenzyme, which blocks Rho activity, completely abolished al-LPA-induced S473 phosphorylation (Fig. 5F). Together, these results suggest that al-LPAs stimulate the same or similar signaling pathways in HEY cells as acyl-LPAs, and they may activate the same or similar receptors.

Activation of MEK/ERK, but not Akt, was required for promoting DNA synthesis by al-LPAs in HEY cells

To explore the potential signaling pathways involved in al-LPA induced DNA synthesis, we tested the effect of PTX, LY294002, wortmannin, PD98059, and SB203580 on [^{3}H]thymidine incorporation induced by alkyl- and alkenyl-LPAs (**Fig. 6A**). PTX inhibited approximately 70% and 45% of [^{3}H]thymidine incorporation triggered by alkyl-LPA and alkenyl-LPA, respectively, suggesting that both PTX-sensitive and insensitive G proteins are involved in this activity. LY294002 (10 μM), wortmannin (100 nM), and PD98059 (30 μM) completely blocked the al-LPA-stimulated DNA synthesis, suggesting that the activity of PI3K and MEK is essential for the process. In contrast, p38 activity was not required for DNA synthesis induced by al-LPAs, since [^{3}H]thymidine incorporation was insensitive to the treatment of SB203580. This was further confirmed by transfection with MEK/2A and p38/AGF (Fig. 6B). Expression of MEK/2A completely inhibited al-LPA-induced DNA synthesis (Fig. 6B). In contrast, expression of p38/AGF did not affect the DNA synthesis induced by al-LPAs (Fig. 6B), indicating that p38 was not required for DNA synthesis induced by al-LPAs. Since S473 phosphorylation of Akt required p38 activation (Fig. 5D), and p38 was not required for the DNA synthesis stimulated by al-LPAs, we predict that Akt activation was not required for al-LPA-induced DNA synthesis. To test this, we transfected the dominant negative (dn) form of Akt into HEY cells and found that dn-Akt did not affect [^{3}H]thymidine incorporation induced by al-LPAs as we predicted (Fig. 6B). These data suggest that al-LPA-induced MEK activation can lead to a p38- and Akt-independent stimulation of DNA synthesis in HEY cells.

Al-LPAs promoted ovarian cancer cell migration through collagen I-coated membranes

Cell migration is critically important for tumor metastasis. Acyl-LPA has been shown to induce cell migration of a number of cell types (fibroblasts, monocytes, T-lymphoma, hepatoma, and endothelial cells) (40–48). To test the effect of LPAs on ovarian cancer cell migration, we conducted Boyden chamber analyses. We found

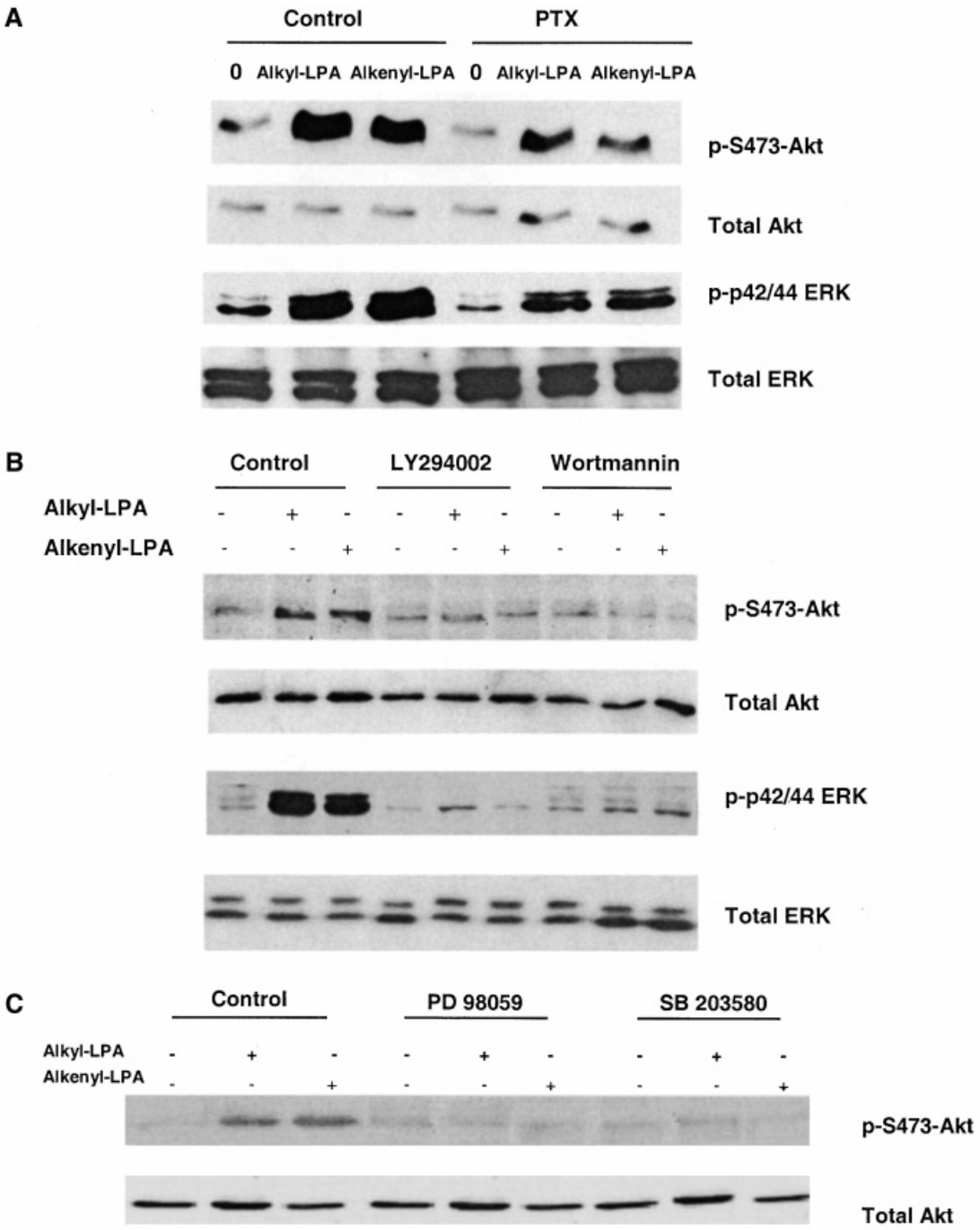

Fig. 5. Al-LPAs induced phosphorylation of ERK, and Akt was dependent on G_i, PI3K, MEK, and p38. A: Akt and ERK phosphorylation induced by al-LPAs was pertussis toxin (PTX)-sensitive. HEY cells were pretreated with PTX (100 ng/ml) for 16 h prior to stimulation with lipids (2 μM) for detection of p-Akt (30 min stimulation) or p-p42/44 ERK (5 min stimulation). B: Al-LPA-induced Akt and ERK phosphorylation was inhibited by PI3K inhibitors. Starved HEY cells were pretreated with 10 μM LY 294002 or 0.1 μM wortmannin for 30 min prior to stimulation with lipids (2 μM; 30 min for p-Akt and 5 min for p-ERK). C: S473 phosphorylation of Akt induced by al-LPAs was dependent on both MEK and p38 MAP kinases. Starved HEY cells were pretreated with 30 μM PD98059 or 10 μM SB203580 for 30 min followed by stimulation with lipid (2 μM; 30 min for p-Akt and 5 min for p-ERK). D: HEY cells were transiently transfected with control vector, dominant negative MEK (MEK/2A), or kinase dead p38 (p38/AGF), and then treated with 2 μM al-LPAs for 30 min. E: HEY cells were pretreated with different concentrations of SB 203580 (0, 0.5, 2.5, and 10 μM) for 30 min, followed by al-LPAs (2 μM) for 30 min. T308 and S473 phosphorylated Akt were detected by specific antibodies. F: S473 phosphorylation of Akt induced by al-LPAs was dependent on Rho activity. HEY cells were transiently transfected with control vector, C3-exoenzyme (C3), and then treated with 2 μM al-LPAs for 30 min.

that both alkyl- and alkenyl-LPAs triggered cell migration through collagen I in a concentration-dependent manner, and alkenyl-LPA was more potent than alkyl-LPA (**Fig. 7A**). To determine whether the enhanced cell migration was due to chemokinesis (random motility) or chemotaxis (directional motility), checkerboard analy-

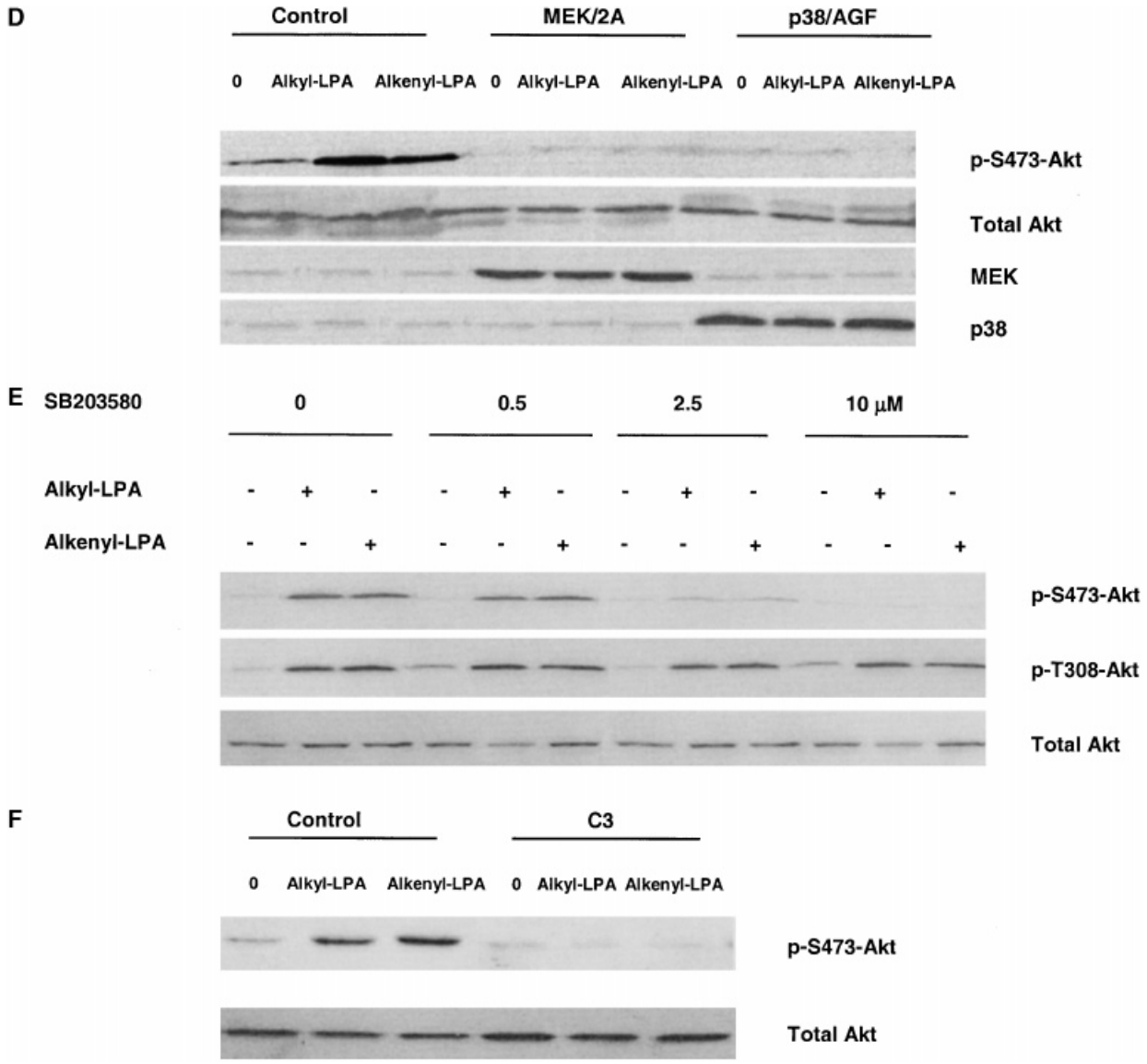

Fig. 5. (*Continued*)

ses were performed essentially as described by Okamoto et al. (37). The number of cells that migrated to the lower phase of the membrane was reduced significantly as the concentration gradient of al-LPAs decreased (**Table 2**), indicating that al-LPAs mainly stimulated chemotaxis.

We then compared the relative potencies of major LPA species present in ascites in stimulation of cell migration. We found that 16:0-acyl, 18:0-acyl, 18:1-acyl, 16:0-alkyl, and 16:0/18:0-alkenyl LPAs all stimulated migration of HEY ovarian cancer cells through collagen I-coated membranes (Fig. 7B). At 1 μM concentration, the relative potencies of these LPA species were 18:1-acyl-LPA > 16:0/18:0-alkenyl-LPA > 16:0-alkyl-LPA > 16:0-acyl-LPA > 18:0-acyl-LPA (Fig. 7B). The cell migration induced by al-LPAs was sensitive to PTX pretreatment and C3 exoenzyme transfection, and partially blocked by LY294002 (Fig. 7C). Interestingly, transfection of MEK/2A, which completely blocked al-LPA-induced Akt phosphorylation (Fig. 5D, top), did not significantly affect cell migration induced by al-LPAs, suggesting that a different downstream signaling molecule(s) of G_i, Rho, and/or PI3-K (other than MEK) was responsible for cell migration induced by al-LPAs.

Al-LPAs triggered IL-8 secretion from HEY cells

We have recently shown that 18:1-acyl-LPA induces increased IL-8 at both mRNA and protein levels in ovarian cancer cells, but not in immortalized ovarian epithelial cells (24). To determine whether al-LPAs also induce this activity, we examined IL-8 secretion from HEY cells using an ELISA assay as previously described (24). Al-LPAs induced IL-8 secretion from ovarian cancer cells with similar or higher potencies to that of 16:0- or 18:0-acyl-LPAs (**Fig. 8**).

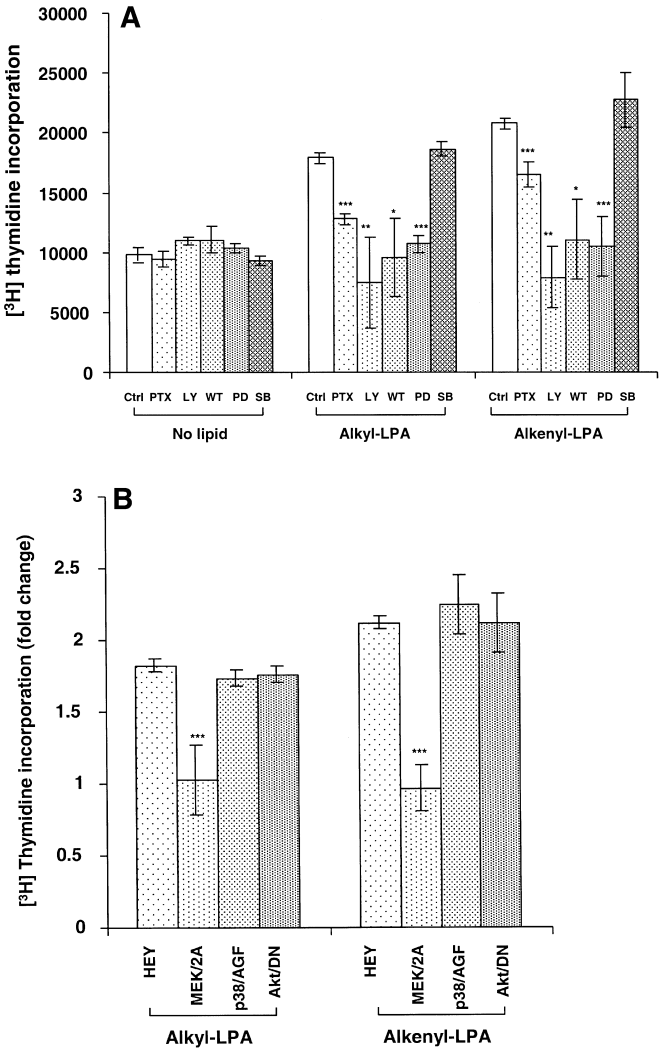

Fig. 6. Al-LPA-mediated proliferation was PTX-sensitive and dependent on PI3K and ERK activation, but not p38 MAP kinase. A: HEY cells were treated with alkyl-, alkenyl-LPAs, or solvent (control), and [^{3}H]thymidine incorporation was conducted as described in Materials and Methods. PTX (100 ng/ml) was added to the culture 16 h prior to lipid (5 μM) stimulation. HEY cells were stimulated with alkyl-LPA or alkenyl-LPA (5 μM) in the presence of 30 μM PD 98059 (PD), 10 μM LY294002 (LY), 0.1 μM wortmanin (WT), or 10 μM SB 203580 (SB). The data shown here represent the mean ± SD of three independent experiments. B: HEY cells were transiently transfected with control vector, dominant negative MEK (MEK/2A), kinase dead p38 (p38/AGF), or dominant negative Akt (AKT/DN). After the starvation, the cells were incubated with 5 μM al-LPAs for 24 h. Results are plotted as mean ± SD of three independent experiments. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ (Student's *t*-test).

DISCUSSION

We have previously reported that acyl-LPAs are growth stimulating factors for ovarian cancer and other tumor cells, which are present in ascites from patients with ovarian cancer (21, 22). The major acyl-LPA species (approximately 50% of all acyl-LPAs) in ovarian cancer ascites is 16:0-acyl-LPA (20). However, it is not a potent growth stimulator of ovarian cancer cells (21, 22). LPA species with unsaturated fatty acids, such as 18:1- and 18:2-acyl LPAs, are more potent mitogens for ovarian cancer cells (20). We have recently detected elevated levels of al-LPAs in ovarian cancer ascites.

In this work, we show several lines of evidence to suggest that al-LPAs may play an important pathological role in ovarian cancer development. First, al-LPAs stimulated cell growth and DNA synthesis of HEY ovarian cancer cells (Fig. 3). Second, al-LPAs induced Akt activation (Fig. 4A), which may be related to cell survival and chemoresistance. Third, al-LPAs induced cell migration (Fig. 7A), which is one of the critical steps in tumor cell invasion and metastasis. Finally, al-LPAs stimulated the production of IL-8 with similar or higher potencies than 16:0- and 18:0-acyl-LPAs (Fig. 8). In particular, physiological concentrations of al-LPAs were used in this study, and our results support the notion that these lipids may play important pathological roles in ovarian cancer development, although the role of al-LPAs in vivo remains to be further investigated.

Ovarian tumor cells inherently possess a strong metastatic potential to the peritoneum, which is the major cause of death in ovarian cancer patients (49). Preferential adhesion of ovarian epithelial carcinoma cells to migrate through collagen I (vs. collagen IV, fibronectin, laminin, and vitronectin), has been demonstrated, and the ovarian carcinoma micro-environment is rich in collagen I (49). We show here that different LPA species promote cell migration through collagen I-coated membranes and that this activity is potentially important in ovarian cancer pathology.

IL-8 is a pro-inflammatory and pro-angiogenic factor and may be involved in ovarian tumor development (50, 51). Angiogenesis is a critical factor for tumor development, which induces the transition from a limited to a rapid tumor growth via neovascularization (52). High expression of IL-8 mRNA has been detected in clinical specimens of late-stage ovarian carcinomas (53, 54). Ascites/cyst fluid and/or plasma of patients with ovarian cancer contain significantly higher levels of IL-8 compared with patients with benign gynecological disorders (55, 56). We have shown that al-LPAs are elevated in malignant ascites (35). Our results shown here suggest that al-LPAs present in ascites may regulate IL-8 production in vivo.

The results shown here suggest that the biological activities and/or signaling properties of LPA species are not only dependent on the composition of the fatty acid side chain, but also the chemical linkage between the aliphatic chain and the glycerol backbone. Although 16:0- and 18:0-acyl-LPAs are not effective in growth stimulation in ovarian cancer cells (20), 16:0-alkyl- and 16:0/18:0-alkenyl-LPAs stimulated growth and DNA synthesis of HEY ovarian cancer cells. In addition, 16:0- and 18:0-al-LPAs were more potent than 16:0- and 18:0-acyl-LPAs in stimulating cell migration and IL-8 production. Interestingly, various synthetic ether-linked lysophosphatidylcholine compounds inhibit growth of many malignant cells, and clinical trials evaluating their antineoplastic potential have been conducted (57, 58). More recently, synthetic alkyl-LPA deriva-

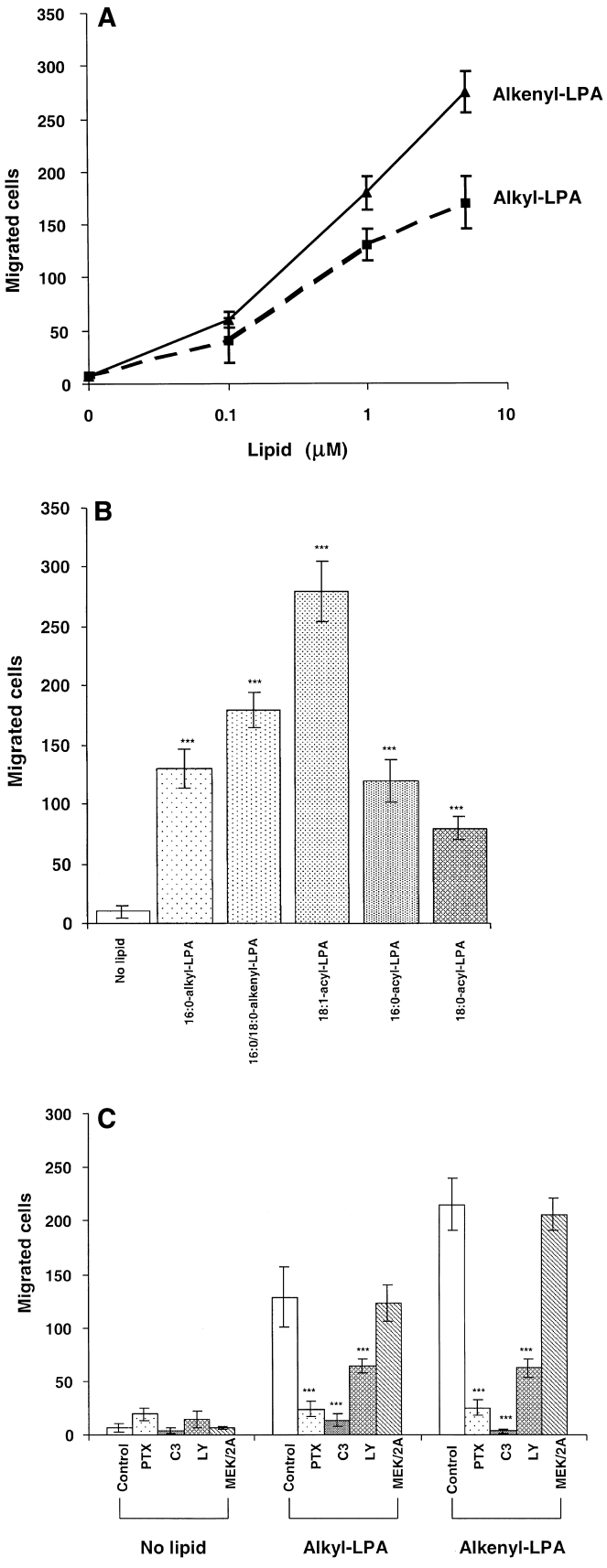

Fig. 7. Al-LPA stimulated HEY cell migration. A: Cell mobility was measured in a modified Boyden chamber assay as described in Materials and Methods. Alkyl- or alkenyl-LPA (0–5 μM) was added to the lower chamber. Cells migrated to the lower phase of the membrane were counted after starved cells were seeded in the upper chamber for 4 h. B: The relative potencies of different LPA species in stimulating cell migration. Different LPA species (1 μM) were added to the lower chamber of the migration chamber, and starved cells were added to the upper chamber. Migration was allowed for 4 h at 37°C. C: Al-LPA-stimulated migration was PTX- and Rho-sensitive and PI3K-dependent. HEY cells were pretreated with PTX (100 ng/ml) for 16 h, or transiently transfected with C3-exoenzyme (C3, a Rho inhibitor), or dominant negative MEK (MEK/2A). HEY cells in the absence (control) or presence of LY294002 (10 μM, LY), as well as PTX pretreated cells or transfected cells, were loaded into the upper chambers and the lipids (1 μM) were added to the lower chamber. The migration was conducted for 4 h. The cell number on the lower face of the membrane was counted. The results are presented as the mean ± SD of three independent experiments. *** $P < 0.001$ (Student's *t*-test).

TABLE 2. Checkerboard analysis of HEY cells[a]

	Alkyl-PLA, Upper Chamber			
	0	0.1	1	5
	μM			
Alkyl-LPA, lower chamber (μM)				
0	68 ± 5	72 ± 13	65 ± 13	55 ± 13
0.1	280 ± 50	167 ± 29	88 ± 10	77 ± 15
1	408 ± 36	367 ± 15	210 ± 30	87 ± 12
5	659 ± 12	587 ± 55	343 ± 21	120 ± 12
Alkenyl-LPA, lower chamber (μM)				
0	78 ± 13	77 ± 8	67 ± 6	82 ± 8
0.1	230 ± 26	150 ± 26	118 ± 28	118 ± 28
1	493 ± 51	293 ± 40	207 ± 15	132 ± 28
5	827 ± 122	550 ± 30	270 ± 72	112 ± 38

[a] Different concentrations of alkyl-LPA or alkenyl-LPA were added to the upper and/or lower chamber, and HEY cells in the upper chamber were allowed to migrate for 4 h at 37°C.

tives have been tested for their anti-proliferative activities (58). Together with the observations present here, these data suggest that a free phosphate group at the *sn*-3 position is important for the mitogenic activity of lysolipid(s).

Acyl-LPAs containing unsaturated fatty acids, such as 18:1- and 18:2-acyl-LPAs, are more potent in stimulation of growth (20), IL-8 secretion, and cell migration. These data suggest that 18:1- and 18:2-acyl LPAs, which compose approximately 17% (Table 1) of total acyl-LPAs in ascites (20, 35) and al-LPAs, which compose approximately 12% of all LPA species, may account for the major portion of biological activities of LPAs in ovarian cancer ascites. The pathophysiological importance of al-LPAs is further supported by our observation that these LPA species are more stable than acyl-LPAs at 4°C (Fig. 1). The instability of LPAs at 4°C may reflect LPA-degrading reactions by endogenous enzymes (at a slower reaction when compared with physiological conditions at 37°C). However, since the ascites samples were stored under sterile conditions, exogenous LPA-degrading enzymes from bacteria and/or other sources were unlikely. The two major pathways to degrade LPA are deacylation by lyso-phospholipase A_1 (PLA_1) and dephosphorylation by phosphatases (LPPs) (59, 60). Though dephosphorylation of al-LPAs and acyl-

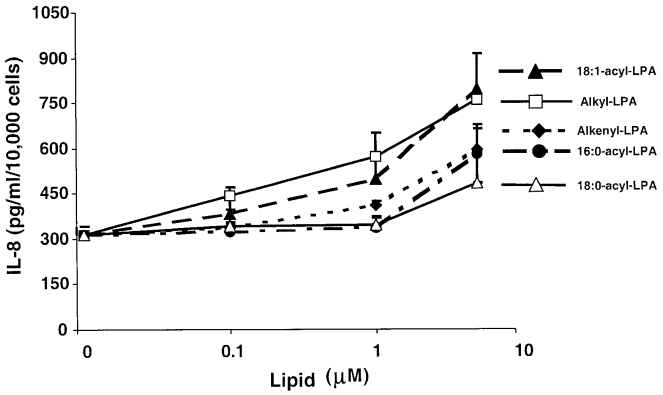

Fig. 8. Stimulation of IL-8 secretion by LPAs in HEY cells. Cells were starved from serum for 18–24 h and treated for 6 h with varying doses of LPAs. The supernatants were then removed and stored in a freezer at −80°C until ELISA (Materials and Methods) was performed.

LPAs by LPPs may be similarly effective, ether-linked al-LPAs are not degradable by PLA_1, which may account for the relatively higher stability of al-LPAs.

We show here that different biological effects induced by al-LPAs require different signaling pathways. PI3K activity is required for cell proliferation, cell migration, and Akt activation/phosphorylation. MEK is required for cell proliferation and S473 phosphorylation of Akt, but not for cell migration. S473 phosphorylation of Akt, but not cell proliferation, is dependent on p38 MAP kinase activity. These data suggest that MEK activation can lead to a p38-dependent Akt phosphorylation, and a p38-independent stimulation of DNA synthesis. These signaling properties provide important information on strategies to antagonize the cellular effects of al-LPAs. Interestingly, we have found that S473, but not T308, phosphorylation induced by al-LPAs is sensitive to SB 203580, suggesting that p38 MAP kinase is required for S473, but not T308, phosphorylation. The involvement of p38 in Akt phosphorylation and activation has been reported in a few recent works (38, 39, 61). In fibroblasts, phosphorylation at both Akt S473 and T308 induced by formyl-methionyl-leucyl-phenylalanine is sensitive to SB 203580, and S473 phosphorylation is more sensitive than T308 to SB 203580. In mouse epidermal J6 cells, UVB-induced T308 phosphorylation is more sensitive than S473 to SB 203580. Together, these and our results suggest that the dependence of T308 phosphorylation on p38 is likely to be stimulus- or cell type-specific.

The work here shows that al-LPAs appear to stimulate the same or similar signaling pathways as acyl-LPAs, although they differ in concentration and time point for optimal stimulations. In particular, we have shown recently that acyl-LPA stimulated a rather unique Rho- and MEK-dependent Akt phosphorylation. This signaling pathway is not shared by many other stimuli that we have tested, including S1P, thrombin, endothelin-1, PDGF, insulin, and EGF (unpublished observations). These data suggest that the effects of al-LPAs may be mediated by acyl-LPA receptors (Edg receptors). In fact, both Edg4 and Edg7 have been shown to respond to alkyl- and/or alkenyl-LPAs (62–64). We have found that HEY cells express Edg2 and 7, and SKOV3 cells express Edg2, 4, and 7 (unpublished observations). Since subtype-selective receptor antagonists are not yet available, the direct assignment of the endogenous receptors mediating the effects induced by al-LPAs in HEY cells remains to be determined.

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