Lysophosphatidic acid induction of urokinase plasminogen activator secretion requires activation of the p38^{MAPK} pathway

VERONICA C. ESTRELLA[1,4*], ASTRID M. EDER[1*], SHUYING LIU[1], TERRI B. PUSTILNIK[2], FAZAL H. TABASSAM[1], FRANCOIS X. CLARET[1,4], GARY E. GALLICK[3,4], GORDON B. MILLS[1,4] and JON R. WIENER[1,4]

Departments of [1]Systems Biology, [2]Gynecologic Oncology, and [3]Cancer Biology, The University of Texas M.D. Anderson Cancer Center; [4]Graduate School of Biomedical Sciences, University of Texas Health Science Center, Houston, TX 77030, USA

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Abstract. Lysophosphatidic acid (LPA) is an important intercellular signaling molecule involved in a myriad of biological responses. Elevated concentrations of LPA are present in the ascites and plasma of ovarian cancer patients suggesting a role for LPA in the pathophysiology of ovarian cancer. We have demonstrated previously that oleoyl (18:1) LPA at concentrations present in ascites induces the secretion of urokinase plasminogen activator (uPA) from ovarian cancer cells, possibly linking LPA to cellular invasion. In this study we sought to elucidate which signaling pathway(s) are involved in LPA-mediated secretion of uPA from ovarian cancer cells. Specific inhibitors were utilized to determine if interference with the p38^{MAPK}, p42/44^{MAPK}, and PI3K pathways functionally blocked LPA-mediated uPA secretion. LPA stimulation of ovarian cancer cells markedly increased the phosphorylation and activity of p38^{MAPK}, p42/p44^{MAPK}, and PI3K. Both tyrosine phosphorylation and Src kinase activity were required for optimal activation of signaling by LPA including phosphorylation of p38^{MAPK}. Inhibition of p38^{MAPK} signaling by SB202190 completely abrogated LPA-induced uPA secretion, while inhibition of the p42/44^{MAPK} or PI3K pathways with PD98059 or wortmannin and LY294002, respectively, decreased but did not completely block uPA secretion. In contrast, inhibitors of phospholipase D or the p70^{S6} kinase pathway did not alter LPA-induced uPA secretion. Further, tyrosine phosphorylation and functional Src were required for optimal uPA secretion. Finally, LPA induces uPA secretion from ovarian cancer cells predominantly through the LPA_2 receptor, with LPA_3 contributing to this process. These results indicate that the p38^{MAPK} signaling pathway is required for optimal LPA-dependent uPA secretion from ovarian cancer cells.

Correspondence to: Dr J.R. Wiener, Graduate School of Biomedical Sciences, University of Texas Health Science Center at Houston, P.O. Box 20334, Houston, TX 77225, USA
E-mail: jon.r.wiener@uth.tmc.edu

*Contributed equally

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Introduction

Lysophosphatidic acid (LPA; 1-acyl-glycero-3-phosphate), the simplest of all the glycerophospholipids, which was originally known as a key intermediate in the biosynthesis of phospholipids, is now recognized as an intercellular phospholipid messenger with a wide range of biological activities, particularly as an inducer of cell proliferation, migration and survival (1,2). LPA is involved in numerous normal biological processes, such as the induction of proliferation of normal fibroblasts (3) and smooth muscle cells (4) and neurite retraction (5,6), wound healing as a result of its involvement in blood clotting, inflammation, and fibronectin binding, and some disease-related processes, including atherosclerosis and cancer (7-9). In ovarian cancer cells, LPA increases proliferation, promotes survival by preventing apoptosis and anoikis, decreases sensitivity to cisplatin, increases activation of MMP1 and MMP2, and increases production of LPA, uPA, VEGF, IL-6 and IL-8 (10-13).

LPA is not present in detectable levels in human plasma from normal subjects, but is produced and released by activated platelets, and is a normal and abundant constituent of human serum present at concentrations of 1-5 μM (14). In contrast, LPA levels are elevated in plasma from women with ovarian cancer (15), and LPA is present at high levels in the ascitic fluid of advanced stage ovarian cancer patients with concentrations of 5-200 μM (16,17). In both human serum and ascitic fluid, it is found in an albumin-bound form, with oleoyl and palmitoyl-LPA being the predominant species (16,18). The strongest support for a link between LPA and cancer stems from the finding that the metastasis-promoting factor autotaxin is an LPA-producing enzyme (19). Importantly, removing LPA by overexpressing lipid phosphate phosphatase inhibits tumor metastasis (20).

LPA specifically binds to at least four distinct G protein-coupled receptors (GPCR) in mammals (2). The most characterized LPA receptors are LPA_1, LPA_2 and LPA_3 which belong

to the Edg (endothelial differentiation gene) subfamily of the GPCR family. A recently identified fourth LPA receptor (GPCR23, p2y9, LPA_4) is closely related to purinergic receptors, but does not share significant identity with the other LPA receptors. LPA receptor gene targeting studies have begun to elucidate the role of LPA in normal physiology with LPA_1-null mice showing defects in olfaction (21). Mice lacking LPA_2 appear phenotypically normal (22), and LPA_1/LPA_2 double-knockout mice show the same abnormalities as LPA_1-null animals (22). LPA_1 is the most widely expressed and best-characterized receptor subtype, whereas LPA_2 and LPA_3 have a more restricted expression pattern (2). Most ovarian cancer cells have higher levels of LPA_2 and LPA_3 expression than normal ovarian surface epithelial cells (23). Nearly all mammalian tissues and organs (except the liver) express at least two LPA receptors of the Edg family (9). In most systems, LPA_2 increases the production of neo-vascularizing factors more efficiently than LPA_1 or LPA_3. Indeed, LPA_2-transgenic ovaries produced significantly higher levels of type A VEGF and uPA than non-transgenic ovaries and displayed elevated VEGF receptor levels (23). LPA_1 seems to be the main receptor regulating cellular motility (9). Binding of LPA to its receptors stimulates the activation of multiple intracellular signaling pathways, including the p42/ 44^{MAPK} mitogen-activated protein kinase (10), c-Jun kinase (24), and PI3K (10) pathways as well as the activation of protein tyrosine kinases (25,26) and, specifically, the Src protein tyrosine kinase (27). These pathways are vital for mediating the signaling events that are involved in such diverse normal cellular functions as responses to extracellular stimuli (28) and differentiation (29).

We have previously reported that LPA is involved in stimulating expression and secretion of urokinase plasminogen activator (uPA) from ovarian cancer cells (30). One of the normal functions of uPA is that of a serine protease involved in the degradation of the basement membrane in the extracellular matrix, resulting in normal cellular migration and tissue remodeling (31). However, in addition to its normal functions, uPA also contributes to the invasive potential of tumor cells during metastasis. Indeed, increased uPA expression is associated with a poor prognosis in ovarian cancer (32,33).

In an effort to determine the mechanism of LPA-induced uPA expression and secretion, we assessed which signaling pathways are stimulated by LPA in human ovarian cancer cells, and whether these pathways are involved in the induction of uPA secretion. In this study we report that LPA induces rapid and transient increases in the levels of activated p38^{MAPK}, PI3K, and p42/44^{MAPK}, but not JNK in ovarian cancer cells. Inhibition of these pathways with specific inhibitors blocked the secretion of uPA to varying extents, indicating that all three pathways are involved in the induction of uPA secretion. However, inhibition of p38^{MAPK} activation completely abrogated uPA secretion. We also provide evidence that tyrosine phosphorylation and the Src tyrosine kinase play a role in LPA-induced uPA secretion. These findings suggest that the stimulation of ovarian cancer cells by LPA results in the secretion of uPA through the activation of multiple signaling pathways, but activation of the p38^{MAPK} pathway appears to be essential.

Materials and methods

Reagents. Lysophosphatidic acid (18:1; oleoyl) was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). The p38^{MAPK} inhibitors SB203580 and SB202190, the MEK1 inhibitor PD98059, the tyrosine kinase inhibitor herbimycin A, the p70^{S6} kinase inhibitor rapamycin, and the PI3K inhibitor LY294002 were obtained from Calbiochem (San Diego, CA). The phospholipase D inhibitor n-butanol, the PI3K inhibitor wortmannin, and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antibodies against human phospho-p38^{MAPK} (Thr180/Tyr182), total p38^{MAPK}, phospho-AKT (Ser473), total AKT, and total MAPK (ERK1 and ERK2) were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal antibody against p38^{MAPK} (C-20-G) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against activated p42/p44^{MAPK} was obtained from Promega Corp. (Madison, WI). Polyclonal antibody against human urokinase was obtained from American Diagnostica Inc. (Greenwich, CT). Anti-BSA antibody was purchased from Upstate (Lake Placid, NY). The single-chain uPA was a generous gift from Dr D. Boyd (The University of Texas M.D. Anderson Cancer Center, Houston, TX).

Cell culture. The OVCAR-3 and SK-OV-3 human ovarian cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA), and maintained as directed. The SK-OV-3 stable clonal transfectants S20 and AS16 were generated by transfection using the pcDNA1 eucaryotic expression vector containing a 22-bp sense or antisense c-*src* oligonucleotide construct, respectively, as described elsewhere (28,29). The SK-OV-3, S20 and AS16 cell lines were maintained in McCoy5A medium/10% FBS, with 400 μg/ml of G418 (Gibco).

LPA stimulation. Cells ($2.5x10^5$) were seeded into 60-mm tissue culture dishes and maintained in complete tissue culture medium until their confluency level was ≅80-90%. Cells were then washed once in serum-free medium (SFM) and starved in 4 ml of SFM for 24 h. The appropriate amount of dried LPA residue was resuspended in 1% delipidated bovine serum albumin (BSA; Sigma) for 30 min on ice. After starvation, the SFM was replaced and the cells were stimulated with 20 μM LPA for 24 h (or as otherwise indicated in figure legends). Cell culture supernatant was collected, concentrated, and analyzed as described below. Monolayers were washed, and lysates prepared and analysed as described below.

Incubation of cells with inhibitors of signal transduction. Cells ($2.5x10^5$) were cultured to ≅80-90% confluency in complete medium, starved for 24 h, and subjected to the appropriate inhibitors for the times indicated in the figures. The final inhibitor concentrations used were 5 μM SB203580, SB202190, and herbimycin A, 1 μM wortmannin, 10 μM LY294002, 0.5 nM rapamycin, and 0.3% n-butanol. Immediately after pre-incubation with inhibitors for various times, cells were stimulated with 20 μM LPA for 24 h (or as described in figure legends). Cellular supernatant and lysates were collected and analyzed as described below.

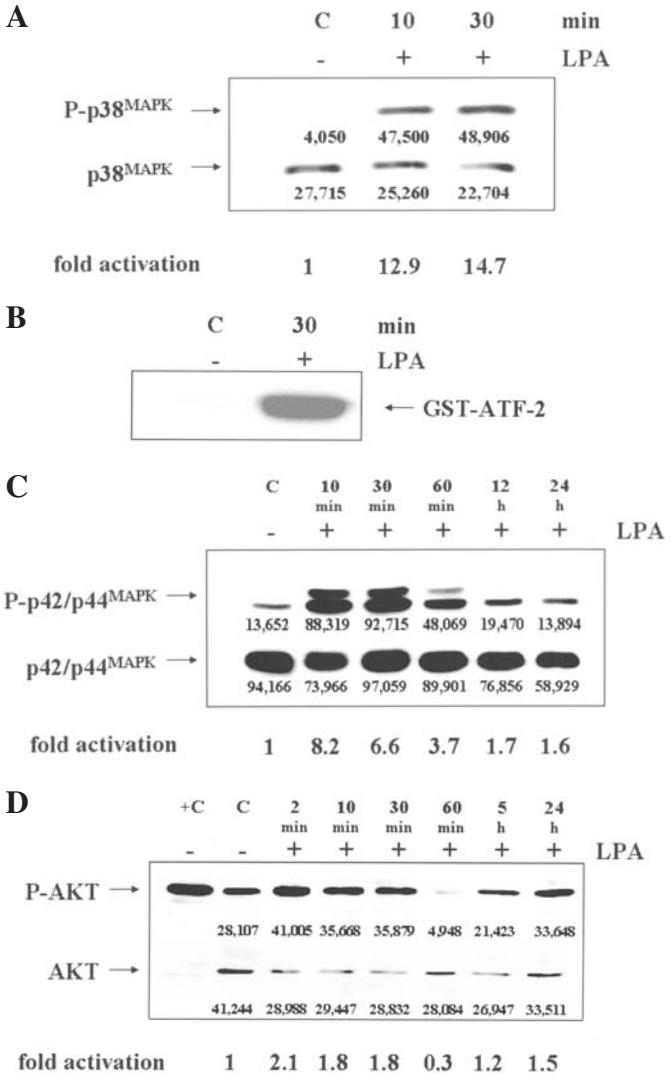

Figure 1. LPA-mediated activation of $p38^{MAPK}$ and $p42/p44^{MAPK}$. A, immunoblot analysis of $p38^{MAPK}$ activation was performed as described in Materials and methods. OVCAR-3 cells were stimulated with 20 μM 18:1 LPA for 10 or 30 min, and lysates from equivalent numbers of cells were electrophoresed and immunoblotted for phosphorylated and activated p38 (P-$p38^{MAPK}$), then stripped and reprobed for total $p38^{MAPK}$. C, control (unstimulated). B, $p38^{MAPK}$ *in vitro* kinase reaction. $p38^{MAPK}$ was immunoprecipitated (3 h at 4˚C) with the polyclonal antibody p38 (C-20)-G and incubated with GST-ATF-2 for 30 min to assess *in vitro* kinase activity. C, control (unstimulated). C, LPA-mediated activation of $p42/p44^{MAPK}$. Immunoblot analysis of $p42/p44^{MAPK}$ was performed as described in Materials and methods. OVCAR-3 cells were stimulated with 20 μM 18:1 LPA for the times shown, and lysates from an equivalent number of cells were electrophoresed and immunoblotted for phosphorylated $p42/p44^{MAPK}$ (P-$p42/p44^{MAPK}$), or total $p42/p44^{MAPK}$. C, control (unstimulated). D, LPA activation of AKT. Immunoblot analysis of AKT activation was performed as described in Materials and methods. OVCAR-3 cells were stimulated with 20 μM 18:1 LPA for the times shown, and lysates from equivalent cell numbers were electrophoresed and immunoblotted for phosphorylated AKT (P-AKT), or total AKT. C, control (unstimulated). +C, PDGF-stimulated NIH3T3 cells (positive control for pAKT).

Immunoblot analysis. Monolayers were lysed in 1X sodium dodecyl sulfate (SDS) Laemmli sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 2.5% bromophenol blue, 5% ß-mercaptoethanol). Cell culture supernatant fluids were harvested and the proteins in the supernatants were fractionated on 12.5% SDS-polyacrylamide gels, electroblotted to Immobilon-P membranes (Millipore, Bedford, MA), and probed with antibodies following the protocols provided by the manufacturers. uPA expression was detected using pretested dilutions of rabbit polyclonal anti-uPA. Immunocomplexes were visualized using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

Zymography. Zymography was performed according to the method of Heussen and Dowdle (30) as described previously. Concentrated cell culture supernatants were resolved by non-reducing SDS-PAGE gels containing plasminogen (Boehringer Mannheim), and then stained with Coomassie brilliant blue R250. The enzymatic conversion of plasminogen to plasmin by uPA was observed as cleared zones in the blue-stained background. Concentrated culture supernatant from UMSCC (neck squamous cell carcinoma) cells (31) was used as a positive control in zymographic gels.

$p38^{MAPK}$ kinase assay. Cell lysates from cells stimulated with LPA were prepared in triton lysis buffer as described (32), and $p38^{MAPK}$ was immunoprecipitated with the polyclonal antibody p38 (C-20)-G. *In vitro* phosphorylation of the $p38^{MAPK}$ substrate GST-ATF2 (residues 1 to 109) was examined after addition of [γ-^{32}P-ATP] as described previously (33). The phosphorylated GST-ATF-2 was detected after SDS-PAGE by autoradiography.

Results

$p38^{MAPK}$, $p42/p44^{MAPK}$ and PI3K are activated by 18:1 LPA in ovarian cancer cells. LPA is known to play a role in stimulating signal transduction events that lead to cell growth and proliferation in fibroblasts and in ovarian and breast cancer cells (16,34). However, which signaling pathways are affected by LPA in ovarian cancer cells, and to what extent individual members of signaling pathways are activated, remains undefined. LPA has been demonstrated to activate $p42/p44^{MAPK}$ through activation of the serine/threonine kinase activity of PI3Kγ (35). Since LPA is a pleiotropic activator of signal transduction pathways in other cell lineages, we examined the possibility that LPA also stimulated the activation of the $p42/p44^{MAPK}$ related kinase, $p38^{MAPK}$, and JNK in OVCAR-3 cells, a malignant human ovarian cancer cell line. Fig. 1A illustrates that LPA induced a rapid and significant increase in the phosphorylated form of $p38^{MAPK}$ following a 10-min treatment with 20 μM 18:1 (oleoyl) LPA. Since phosphorylation activates $p38^{MAPK}$ (36), we directly assessed in an *in vitro* kinase assay whether the observed LPA-induced phosphorylation of $p38^{MAPK}$ coincided with induction of $p38^{MAPK}$ activation, as measured by phosphorylation of the $p38^{MAPK}$ target ATF-2. In agreement with the phosphorylation of $p38^{MAPK}$, the results demonstrated that 30-min treatment with 18:1 LPA rapidly increased the activity of $p38^{MAPK}$ (Fig. 1B). The treatment of OVCAR-3 cells with 20 μM 18:1 LPA also rapidly and transiently stimulated the activation of $p42/p44^{MAPK}$ (Fig. 1C). In agreement with our previous results with other cell types, LPA did not induce detectable activation of the JNK pathway in OVCAR-3 cells (data not shown, 37). Together, these results suggest that LPA stimulation of ovarian cancer cell proliferation (16) coincides with

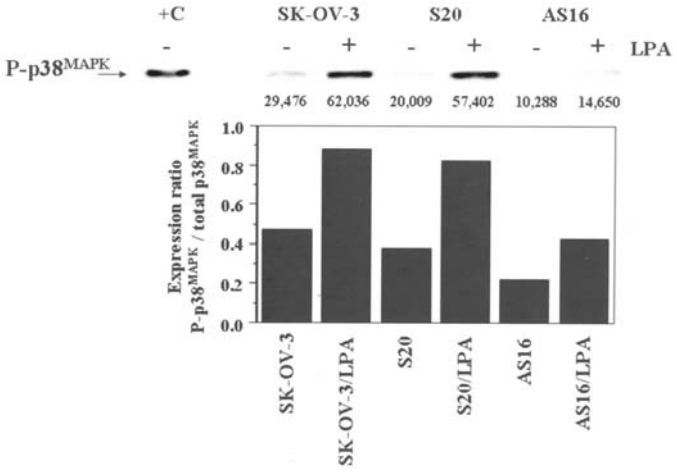

Figure 2. LPA activation of p38^{MAPK} in SK-OV-3 and c-*src* clones. Immunoblot analysis of p38^{MAPK}. SK-OV-3, S20, and AS16 cells were stimulated for 24 h with 20 μM 18:1 LPA. C, single-chain uPA as a positive control. Cell lysates of equal cell number were electrophoresed and immunoblotted for phosphorylated p38^{MAPK} (P-p38^{MAPK}), or total p38^{MAPK}. +C, cellular lysates of sorbitol-stimulated OVCAR-3 cells (positive control for activated p38^{MAPK}). Expression of total p38^{MAPK} was uniform in all samples analyzed. Expression ratio was calculated as densitometry of P-p38^{MAPK}/densitometry of total p38^{MAPK}.

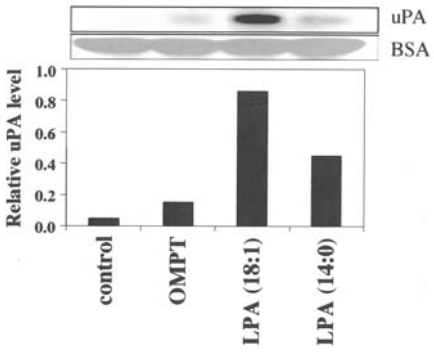

Figure 3. Receptor-selectivity of LPA-induced uPA secretion. OVCAR-3 cells were stimulated for 24 h with OMPT (20 μM), 18:1 LPA (20 μM) and 14:0 LPA (20 μM). BSA was used as control for LPA carrier. Cell culture supernatants were concentrated, and total protein was electrophoresed and immunoblotted for uPA. NIH imager was used for densitometry and quantitation.

activation of p38^{MAPK} and p42/44^{MAPK} signaling. Similar results were observed in the SK-OV-3 ovarian cancer line (data not shown). Finally, we assessed whether LPA induced the activation of phosphatidylinositol 3-kinase (PI3K). Extensive analysis of the PI3K signaling pathway by others has shown that PI3K functions upstream of AKT activation, and that AKT may be a target for PI3K activity (38). To determine whether LPA stimulation induced the activation of PI3K in OVCAR-3 cells, we assessed cell lysates for the appearance of the phosphorylated and thus activated form of AKT following treatment of cells with 20 μM 18:1 LPA. LPA induced rapid and transient AKT phosphorylation by 2 min, followed by a sharp decrease by 60 min, and a subsequent return to baseline levels by 5 h (Fig. 1D). These results suggest that LPA can also stimulate the PI3K signaling pathway in OVCAR-3 cells.

The Src tyrosine kinase is involved in the activation of p38^{MAPK} by LPA. Activation of the Ras/MAPK signaling pathway is often initiated by ligand-mediated dimerization and subsequent activation of receptor protein tyrosine kinases, with subsequent activation of non-receptor tyrosine kinases (39). Indeed, LPA induces tyrosine phosphorylation of multiple substrates in ovarian cancer cells including members of the EGF receptor family (16). Since we had observed that LPA stimulated the phosphorylation and activation of the p38^{MAPK} and p42/p44^{MAPK} signaling pathways, and since LPA-induced p42/p44^{MAPK} activation has been shown previously to be tyrosine phosphorylation-dependent (25,26), we investigated whether tyrosine phosphorylation was also involved in p38^{MAPK} activation. Indeed, LPA-mediated p38^{MAPK} phosphorylation/activation was completely abrogated by pretreatment of OVCAR-3 cells with the nonspecific tyrosine kinase inhibitor herbimycin A (data not shown), suggesting that a tyrosine kinase was involved in the process.

LPA mediates the activation of the Src tyrosine kinase by an as yet undefined mechanism (27). Given the linkage between LPA and activation of the Src tyrosine kinase, we investigated whether Src was involved in the activation of p38^{MAPK} by LPA. Stable clonal transfectants of the SK-OV-3 malignant human ovarian cancer cell line with decreased Src tyrosine kinase activity were generated using expression vectors containing an antisense c-*src* oligonucleotide (40,41). Similar control clonal isolates were obtained using sense constructs. The antisense c-*src* clone AS16 displays >90% reduction in Src tyrosine kinase activity and reduced tumorigenic and angiogenic potential as compared to parental SK-OV-3 and S20 sense cells (41). Stimulation of the parental SK-OV-3 cells as well as the sense c-*src* clone S20 with 18:1 LPA for 30 min induced significant activation of p38^{MAPK} (Fig. 2), in agreement with results observed in the parental OVCAR-3 line. In contrast, basal p38^{MAPK} activity was significantly diminished in the antisense c-*src* clone AS16, and the level of LPA-induced activation of p38^{MAPK} in the AS16 clone was markedly reduced. These findings suggest that the Src tyrosine kinase may play an essential role in basal p38^{MAPK} activation and the level of p38^{MAPK} activation induced by LPA.

LPA activation of p38^{MAPK}, p42/p44^{MAPK}, and PI3K induces the secretion of uPA from ovarian cancer cells. We have shown previously that 18:1 LPA mediates the production and secretion of urokinase plasminogen activator (uPA) from ovarian cancer cells, but not from normal ovarian epithelium (30). Further, LPA mediates uPA secretion from SK-OV-3 and OVCAR-3 cells in a time-, concentration-, and structure-dependent manner (30). 18:1 LPA, but not 18:0 LPA or 16:0 LPA induced uPA secretion from these cells (30). Further, our data indicate the involvement of the LPA_2 receptor in LPA-mediated uPA secretion from OVCAR-3 cells, as 14:0 LPA, which activates LPA_2, but not LPA_1 or LPA_3 (42) also stimulates uPA secretion (Fig. 3). LPA_3, which is selectively activated by the LPA analog OMPT (43), contributes to uPA secretion from OVCAR-3 cells to a lesser extent than LPA_2 (Fig. 3). LPA 18:1 is a pan-activator of LPA receptors. However, the expression pattern of LPA receptors in OVCAR-3 cells is $LPA_3 > LPA_2 > LPA_1$.

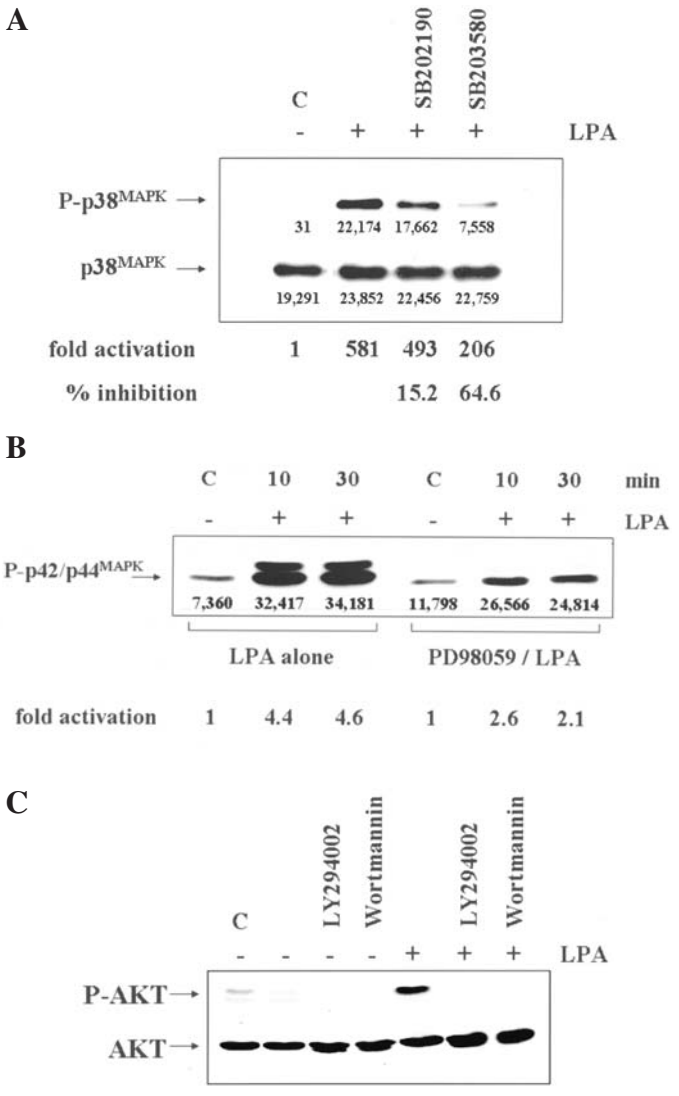

Figure 4. LPA activation of p38^{MAPK}, p42/p44^{MAPK}, and AKT is blocked by the use of specific inhibitors. A, inhibition assay with the p38^{MAPK} inhibitors, SB202190 and SB203580, and immunoblot analysis of p38^{MAPK} activation was performed as described in Materials and methods. Following pre-incubation with 5 μM SB202190 or SB203580 for 1 h, OVCAR-3 cells were stimulated with 20 μM 18:1 LPA for times shown. Cell lysates of equivalent cell number were electrophoresed and immunoblotted for phosphorylated p38^{MAPK} (P-p38^{MAPK}) or total p38^{MAPK}. C, control (unstimulated). B, inhibition of LPA-mediated p42/p44^{MAPK} activation with the MEK1 inhibitor PD98059. Following pre-incubation with 20 μM PD98059 for 24 h, OVCAR-3 cells were stimulated with 20 μM of 18:1 LPA for 10 and 30 min. Lysates containing equal cell numbers were electrophoresed and immunoblotted for phosphorylated p42/p44^{MAPK} (P-p42/p44^{MAPK}). Total p42/p44^{MAPK} expression levels were uniform in all samples analyzed. C, control (unstimulated). C, inhibition of LPA-mediated AKT activation with LY294002 and wortmannin. Following pre-incubation with 10 μM LY294002 and 1 μM wortmannin for 4 h, OVCAR-3 cells were stimulated with 20 μM 18:1 LPA for 30 min. Cell lysates containing equal numbers of cells were electrophoresed and immunoblotted for phosphorylated AKT (pAKT), or total AKT. C, control (unstimulated). DMSO, solvent control.

Since LPA mediated both uPA secretion and activation of various signaling pathways in ovarian cancer cells, we determined which if any of the pathways were involved in uPA secretion. As indicated above, LPA can mediate the activation of p38^{MAPK} (Fig. 1A and B), p42/p44^{MAPK} (Fig. 1C), and PI3K (Fig. 1D). Activation of these molecules can be blocked by the use of specific inhibitors. For example, the stimulation of p38^{MAPK} by LPA was blocked by 65% and 15%, respectively, by two p38^{MAPK}-specific inhibitors, SB203580 and SB202190 (Fig. 4A). The MEK1 (MAPKK) inhibitor PD98059 blocked LPA-induced activation of p42/p44^{MAPK} by 70% (Fig. 4B). The activation of AKT by LPA was completely blocked by inhibiting PI3K activity with LY294002 or wortmannin (Fig. 4C). We subsequently determined whether these inhibitors specifically blocked the LPA-mediated activation of signaling molecules in OVCAR-3 cells. The results indicated that the p38^{MAPK} inhibitors SB203580 and SB202190 specifically inhibited LPA-mediated p38^{MAPK} activation without altering the ability of LPA to induce p42/p44^{MAPK} activation. Similarly, the MEK1 inhibitor, PD98059, effectively blocked LPA-mediated p42/p44^{MAPK} activation without altering LPA-induced activation of p38^{MAPK}. Finally, LY294002 and wortmannin specifically blocked the activation of AKT by LPA without interfering with LPA-induced activation of either p42/p44^{MAPK} or p38^{MAPK} (Table I).

Table I. Specificity of signal transduction pathway inhibitors in OVCAR-3 cells.

	Signaling pathway		
Inhibitor	p42/44^{MAPK}	PI3K	p38^{MAPK}
LY294002	0	++++	0
Wortmannin	0	++++	0
PD098059	++	ND	0
SB203580/SB202190	0	ND	+++

MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3-kinase. 0, no inhibition; ++, 70-80% inhibition, +++, 85-95% inhibition; ++++, complete inhibition. Inhibitor concentration as described in Materials and methods. ND, not determined.

Utilizing these inhibitors, we next examined whether the p42/p44^{MAPK}, p38^{MAPK} or PI3K pathways are required for LPA-mediated uPA secretion. As assessed by Western blotting, the amount of uPA secreted after treatment with LPA was inhibited by 90% in the presence of the p38^{MAPK} inhibitor, SB203580 (Fig. 5A). uPA secretion was completely blocked after treatment with another p38^{MAPK} inhibitor, SB202190. In addition, zymography of concentrated cell supernatants indicated that LPA-induced increases in uPA activity were decreased by 50% after treatment with SB203580 (Fig. 5B). Further, pretreatment with the tyrosine kinase inhibitor herbimycin A completely blocked uPA secretion (Fig. 5A), which indicates that tyrosine phosphorylation is required for LPA-mediated uPA secretion. In addition, uPA secretion was decreased by both the PI3K inhibitor LY294002 (80% inhibition) and the MEK1 inhibitor PD98059 (85% inhibition). The inhibitors rapamycin and n-butanol did not decrease LPA induction of uPA secretion, suggesting that uPA secretion is independent of p70^{S6} kinase and phospholipase D activation, respectively (Fig. 5A). Taken together, these findings suggest that uPA secretion is entirely dependent on the activation of p38^{MAPK} by LPA, and partially dependent on LPA-induced activation of both PI3K and p42/44^{MAPK}.

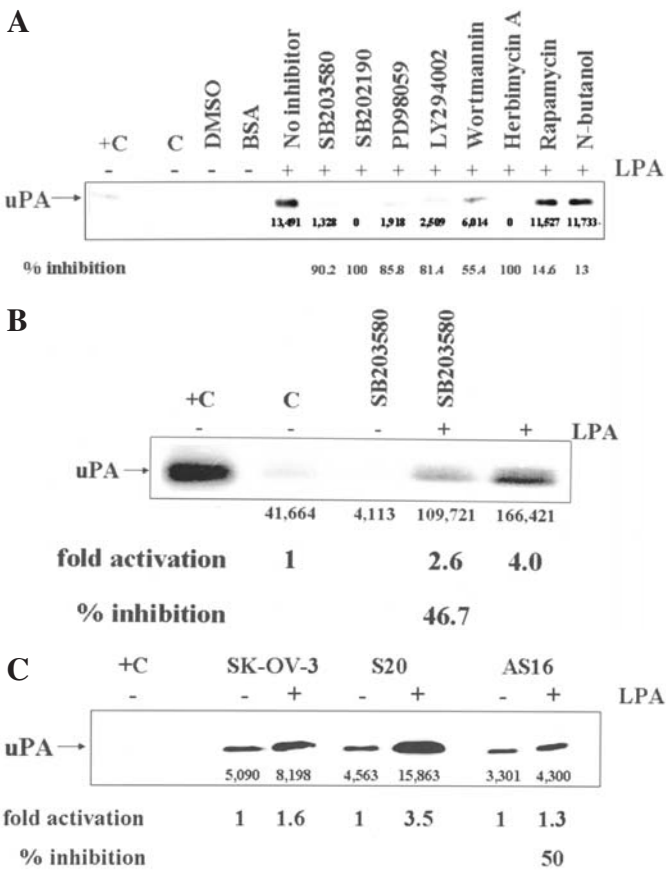

Figure 5. Inhibition of LPA-mediated uPA activation and secretion. A, uPA immunoblot following inhibition assay. Inhibition assay and immunoblot analysis performed as described in Materials and methods. OVCAR-3 cells were pre-incubated with SB202190, SB2023580, LY294002, wortmannin, herbimycin A, rapamycin, and n-butanol for 4 h, and with PD98059 for 24 h. Following pre-incubation, cells were stimulated with 20 μM 18:1 LPA for 24 h. Cell culture supernatants were concentrated and 80 μg of total protein was electrophoresed and immunoblotted for uPA. DMSO and BSA were used as controls for solvent and LPA carrier, respectively. C, control (unstimulated). +C, single-chain uPA (positive control). B, zymogram of uPA activity was performed as described in Materials and methods. Following pre-incubation with 5 μM of the p38^{MAPK} inhibitor, SB203580, for 1 h, OVCAR-3 cells were stimulated with 20 μM 18:1 LPA for 24 h. Cell culture supernatants were concentrated 33-fold and 80 μg of total protein was electrophoresed through gels containing plasminogen. Gels were then stained with Coomassie blue to reveal clear zones, indicative of uPA activity. +C, concentrated culture supernatant from UMSCC (neck squamous cell carcinoma) cells (positive control for uPA activity). C, control (unstimulated). C, immunoblot analysis of uPA. SK-OV-3, S20 and AS16 cells were stimulated for 24 h with 20 μM 18:1 LPA. Cell culture supernatants were concentrated and 80 μg of total protein was electrophoresed and immunoblotted for uPA. +C, single-chain uPA (positive control).

LPA induction of uPA secretion involves the Src tyrosine kinase. The results described above suggest that the Src tyrosine kinase is involved in the activation of p38^{MAPK} by LPA. Taken together with our observation that LPA-stimulated uPA secretion requires p38^{MAPK} activation in ovarian cancer cells, we next determined whether Src kinase activity was involved in LPA-mediated uPA secretion. We therefore assessed LPA-mediated uPA secretion in the SK-OV-3 cell line engineered to possess reduced Src activity (Fig. 2). SK-OV-3 parental cells, control c-*src* sense S20 cells, and the antisense c-*src* line AS16 were starved, and then stimulated with 20 μM LPA (18:1) for 24 h. Both the SK-OV-3 parental and S20 cell lines were responsive to LPA stimulation, as indicated by significant increases in uPA secretion. In contrast, LPA-induced uPA secretion was decreased by 50% in the AS16 cell line (Fig. 5C). These results suggest that the Src tyrosine kinase not only plays a role in the activation of p38^{MAPK}, but may also be essential for the LPA-mediated secretion of uPA from ovarian cancer cell lines.

Discussion

LPA, the simplest phospholipid, is a multifunctional signaling molecule that is involved in several important biological responses including cell proliferation, platelet aggregation, fibroblast growth, and wound repair (8). LPA is a normal constituent of human serum but not of human plasma (44). However, it is present in significant concentrations in the plasma of early-stage human ovarian cancer patients, and may represent a novel tumor marker for early detection (15). LPA is also present in high concentrations in the ascites of late-stage ovarian cancer patients (16,17). Due to its ability to act as a mitogen and promote the activation and proliferation of ovarian cancer cells, it was originally termed ovarian cancer growth factor or OCAF (16).

LPA induces signaling by associating with specific cell surface receptors that couple to heterotrimeric G proteins (2), activating a variety of signaling molecules, such as Ras, Rho, MAPK, Src, and PI3K (2,10,27). A complete understanding of the molecular mechanisms through which LPA regulates these signaling events has not yet been ascertained. We have reported that physiologically relevant concentrations of LPA induce the secretion of uPA from ovarian cancer cells in a time-, structure-, and concentration-dependent manner (30). uPA is a potent serine protease capable of degrading the extracellular matrix and has been implicated in cancer cell invasion and metastasis (45). Benign ovarian tumors express low levels of uPA whereas uPA expression and activity are significantly increased in advanced ovarian tumors (46-49). Indeed, the presence of high levels of uPA in ovarian tumor samples correlates with poor prognosis (50,51). UPA induces proliferation (52,53) and migration (54) of ovarian cancer cells. Inhibiting uPA function decreases ovarian cancer invasion and metastasis (55-57). Since LPA stimulates both cell signaling and the secretion of a prognostically significant protease involved in cancer cell metastasis, it was of interest to explore further which signaling pathway(s) was involved in production and secretion of uPA from ovarian cancer cells.

To this end, we analyzed whether uPA secretion was dependent on LPA-mediated stimulation of the p42/p44^{MAPK}, p38^{MAPK} and PI3K signaling pathways. Our results indicate that short-term stimulation by LPA is capable of inducing a rapid and significant increase in phosphorylation and kinase activity of p38^{MAPK}. In contrast, LPA did not induce the phosphorylation of JNK. These findings are in agreement with previous studies from this laboratory (37) as well as with studies performed in human hepatoblastoma cells which indicate that LPA causes a slight increase in p38^{MAPK} activity but fails to activate JNK (58). In agreement with studies in other lineages (59) and our studies in ovarian cancer cells (16), LPA activated p42/p44^{MAPK} in ovarian cancer cells. These results suggest that LPA initiates a signaling cascade(s) that

activates both p38^{MAPK} and p42/p44^{MAPK} in ovarian cancer cells.

Various isoforms of PI3K exist and, under some circumstances, are involved in LPA-induced signal transduction and activation of the Ras/MAPK pathway (44). PI3Kγ links the LPA receptor to the Ras/MAPK signaling pathway (60). PI3Kß is required for LPA-mediated signal transduction during mitogenesis in fibroblasts (61). Our studies revealed that after LPA treatment, phosphorylation levels of AKT were significantly elevated indicating concomitant activation of PI3K. Further, we observed that LPA-induced p42/p44^{MAPK} activation was not blocked after treatment of cells with the PI3K inhibitors wortmannin and LY294002 which completely inhibited PI3K as indicated by AKT phosphorylation. These results suggest that LPA-mediated PI3K activation is not an obligatory step in LPA-induced p42/p44^{MAPK} activation in ovarian cancer cells.

Our previous studies have shown that oleoyl (18:1) LPA stimulates the production and secretion of significant quantities of uPA from ovarian cancer cells at least in part by activating *de novo* transcription from the uPA promoter (30). We have extended these studies and show here that in ovarian cancer cells, LPA_2 is the dominant receptor involved in LPA-induced uPA production and secretion, with some participation of LPA_3. Both LPA_2 and LPA_3 are overexpressed in ovarian cancer cells as compared to normal ovarian surface epithelial cells. To investigate the signaling pathway(s) that may be involved in LPA-mediated uPA production and secretion, we utilized specific inhibitors of various signal transduction pathways and assessed LPA-mediated pathway activation and induction of uPA expression and secretion from ovarian cancer cells. Our results indicated that the p38^{MAPK} inhibitors SB202190 and SB203580 partially blocked LPA-mediated p38^{MAPK} activation and completely blocked uPA secretion. The MEK1 inhibitor PD98059 partially blocked both LPA-mediated p42/p44^{MAPK} activation and uPA secretion. Finally, the PI3K inhibitors LY29402 and wortmannin completely blocked PI3K activation, and partially blocked uPA secretion. We conclude that the p38^{MAPK} pathway is essential and perhaps dominant for LPA-mediated uPA secretion from ovarian cancer cells, while activation of the p42/p44^{MAPK} and PI3K pathways contributes to, but is not obligatory for uPA secretion. Our data are in agreement with previously published studies that showed a critical role for p38^{MAPK} in uPA expression in different systems. In endothelial cells, the p38^{MAPK} pathway participates in migration by regulating uPA expression (62). In invasive breast cancer cells, vimentin-ligation of αv integrin results in p38^{MAPK} activation and subsequent upregulation of uPA expression (63). In contrast to our findings, a recent study did not find any contribution of the p42/p44^{MAPK} and PI3K pathways to LPA-induced uPA secretion (64). The conflicting data may be due to the use of different cell lines.

The intermediate steps that link G protein-coupled receptors, such as LPA receptors, to p38^{MAPK} or Ras/p42/p44^{MAPK} activation, are not well defined. Tyrosine kinases such as Src and Pyk2 have been linked to the activation of p42/p44^{MAPK} via G protein coupled receptors (27,65). We therefore addressed the possible involvement of Src in LPA-mediated induction of uPA secretion. Our results showed diminished uPA secretion from SK-OV-3 cells expressing reduced Src levels and enzyme activity. These results suggest a role for Src in LPA-induced uPA secretion from ovarian cancer cells. We hypothesize that Src may be upstream of the Rac/p38^{MAPK} or the Ras/p42/p44^{MAPK} cascades involved in LPA-mediated uPA secretion. In support of this contention, LPA-induced p38^{MAPK} and p42/p44^{MAPK} phosphorylation, and likely activation, were markedly decreased in cells with diminished Src levels and activity. Interestingly, Src also plays a critical role in TGF-ß1-induced uPA gene expression in ovarian cancer cells (66). Further, we show that the tyrosine kinase inhibitor herbimycin A completely blocks LPA-mediated uPA secretion suggestive of an essential role of tyrosine phosphorylation in that process, possibly mediated by Src. Activation of both G protein-coupled receptors (LPA receptors) and receptors with intrinsic tyrosine kinase activity, such as the insulin-like growth factor (IGF) I receptor, results in elevated uPA levels. Both types of receptors seem to employ similar signaling pathways. Induction of both uPA mRNA and protein by IGF-I is partially blocked by MEK1 and PI3K inhibitors, whereas herbimycin A also completely blocks IGF-I-mediated uPA expression (67).

Our results do not exclude a role for p38^{MAPK}, p42/p44^{MAPK} or PI3K in posttranscriptional regulation of uPA mRNA. A previous study showed posttranscriptional regulation of uPA by p38^{MAPK} in the MDA-MB-231 breast cancer cell line (68). Inhibition of p38^{MAPK} resulted in degradation of uPA mRNA, which was mediated by an AU-rich sequence present in the 3' untranslated region of the uPA mRNA, but did not affect uPA gene transcription.

In conclusion, our results show that in ovarian cancer cells, LPA induces the activation of the signaling molecules p42/p44^{MAPK}, p38^{MAPK} and PI3K. We show that p38^{MAPK} activity is required for LPA-mediated uPA secretion from ovarian cancer cells, with p42/p44^{MAPK} and PI3K activities contributing to this process. The involvement of p38^{MAPK} seems to be dominant over the roles that p42/p44^{MAPK} and PI3K play in inducing LPA-dependent uPA secretion. Our results indicate that neither the phospholipase D nor the p70S6 kinase pathways are involved.

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