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# Lysophosphatidic acid induces cell migration through the selective activation of Akt1

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Abbreviations: ALCP, ascites from liver cirrhosis patients; AOCP, ascites from ovarian cancer patients; GPCR, G protein coupled receptor; LPA, 1- or 2-acyl-*sn*-glycero-3-phosphate; MEF, mouse embryo fibroblast; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; P-Rex1, $PIP_3$-dependent Rac exchanger1

## Abstract

**Akt plays pivotal roles in many physiological responses including growth, proliferation, survival, metabolism, and migration. In the current studies, we have evaluated the isoform-specific role of akt in lysophosphatidic acid (LPA)-induced cell migration. Ascites from ovarian cancer patients (AOCP) induced mouse embryo fibroblast (MEF) cell migration in a dose-dependent manner. On the other hand, ascites from liver cirrhosis patients (ALCP) did not induce MEF cell migration. AOCP-induced MEF cell migration was completely blocked by pre-treatment of cells with LPA receptor antagonist, Ki16425. Both LPA- and AOCP-induced MEF cell migration was completely attenuated by PI3K inhibitor, LY294002. Furthermore, cells lacking *Akt1* displayed defect in LPA-induced cell migration. Re-expression of Akt1 in DKO (*Akt1*$^{-/-}$*Akt2*$^{-/-}$) cells restored LPA-induced cell migration, whereas re-expression of Akt2 in DKO cells could not restore the LPA-induced cell migration. Finally, Akt1 was selectively phosphorylated by LPA and AOCP stimulation. These results suggest that LPA is a major factor responsible for AOCP-induced cell migration and signaling specificity of Akt1 may dictate LPA-induced cell migration.**

**Keywords:** ascites; cell movement; fibroblasts; lysophosphatidic acid; 1-phosphatidylinositol 3-kinase; proto-oncogen proteins c-akt

## Introduction

Cell migration is essential for normal embryonic development, immune system function, angiogenesis, but it is also associated with inflammatory disease, vascular impairment, and tumor cell invasion (Lauffenburger and Horwitz, 1996; Ridley *et al*., 2003; Raftopoulou and Hall, 2004). Both the speed and the directionality of cell migration are mediated by complex mechanism that includes the formation of membrane protrusions such as lamellipodia and membrane adhesive interactions with the migratory substrate, and coordinated dynamics of cytoskeletal rearrangement (Lauffenburger and Horwitz, 1996; Ridley *et al*., 2003). Directional migration appears to be regulated by multiple mechanisms, including microtubules, CDC42, integrins, and chemotactic stimuli (Weiner, 2002; Dujardin *et al*., 2003; Etienne-Manneville and Hall, 2003). Chemotactic stimulation results in the local activation of phosphatidylinositol 3'-kinase (PI3K) and establishment of phosphoinositide (PIP3) gradient which eventually leads to the local activation of Rac or Ras (Srinivasan *et al*., 2003; Sasaki *et al*., 2004). The site of PIP3 accumulation at the leading edge allows cells to localize a number of integrated signaling complexes that control forward movement, including RacGEFs and Rac/CDC42 effectors such as Wiskott-Aldrich syndrome protein (WASP) and WASP-family verprolin homologous protein (WAVE) (Han *et al*., 1998; Russo *et al*., 2001; Oikawa *et al*., 2004).

Lysophosphatidic acid (LPA, 1- or 2-acyl-*sn*-glycero-3-phosphate) is a phospholipid ligand that normally exists in serum and body fluids. LPA is regarded as a biomarker for ovarian cancer, and a high level of LPA is detected in ascitic fluids as well

as the plasma of ovarian cancer patients (Xu *et al*., 1998). It has been reported that LPA stimulates cell migration in many cell types, including endothelial cells and some fibroblasts (Pietruck *et al*., 1997; Panetti *et al*., 2000). In addition, LPA has also been suggested to be involved in tumor cell migration, such as in ovarian cancer cells and rat hepatoma cells (Imamura *et al*., 1999; Fishman *et al*., 2001).

LPA exerts its biological function by interacting with the G protein-coupled receptors (GPCRs) $LPA_1$/Edg-2, $LPA_2$/Edg-4, and $LPA_3$/Edg-7 (Contos *et al*., 2000). Occupation by LPA in its cognate receptors triggers activation of various signaling molecules during cell migration. For example, LPA activates the Ras/MEKK/MAPK pathway, p38 MAPK, and JNK (Bian *et al*., 2004; Malchinkhuu *et al*., 2005). It has been reported that PI3K is also activated by LPA stimulation through two different mechanisms. LPA evokes the activation of class I PI3K through either the transactivation of the EGF receptor or direct activation of class II PI3K (Maffucci *et al*., 2005; Shah *et al*., 2006).

Akt protein kinases are major downstream effectors of PI3K. The mammalian Akt enzyme family consists of three different isozymes that are encoded by three different genes (Datta *et al*., 1999). Recent studies have demonstrated that each isoform of Akt plays distinct roles in proliferation, metabolism, and survival (Cho *et al*., 2001a; Cho *et al*., 2001b; Bae *et al*., 2003). However, it is still not clear whether LPA-induced cell migration is also regulated by an Akt isoform-specific mechanism. Here, we provide evidence that Akt1 is the main isozyme responsible for LPA-induced cell migration.

## Materials and Methods

### Reagents

All cell culture media and supplements were purchased from Cambrex corp. Anti-pan-Akt antibody, anti-Akt1 antibody, anti-Akt2, anti-phospho-Akt (Ser473), and anti-myc epitope antibodies were purchased from Upstate Biotech. ChemoTx membrane (8 μm pore size) was obtained from Neuro Probe Inc. Lysophosphatidic acid (LPA, 1- or 2-acyl-*sn*-glycero-3-phosphate) and all other high quality reagents were purchased from Sigma-Aldrich unless otherwise indicated.

### Establishment of mouse embryo fibroblast (MEF) cells and culture

Primary MEF cells were isolated from embryos of pregnant female ($Akt1^{+/-}/Akt2^{+/-}$) mice that had been bred with male ($Akt1^{+/-}/Akt2^{+/-}$) mice (Bae *et al*., 2003). The yolk sacs, heads, and internal organs were isolated and used for genotyping by RT-PCR. Carcasses were treated with trypsin-EDTA for 30 min at 37°C, and clumps of cells were disrupted by chopping with scissors. After centrifugation, the cells were re-suspended in culture medium (DMEM supplemented with 10% FBS and antibiotics) and primary MEF cells were immortalized by continuous culturing for 30 passages.

### Plasmids construction

Myc-tagged murine *Akt1* and *Akt2* were cloned into a retroviral vector, pMIGR2, as described previously (Zhou *et al*., 2006). All constructs were fully sequenced to ensure that no other mutations were introduced inadvertently.

### Gene expression by retroviral infection

Generation and infection of retroviral particles for the expression of Akt genes were performed essentially in the same manner as previously described (Bae *et al*., 2003). Briefly, cells were triple transfected with 10 μg of pMIGR2 retroviral constructs, 2 μg of pGag/Pol, and 2 μg of pVSV-G by calcium phosphate method. Medium was replaced with fresh medium 8 h post-transfection. Retroviral supernatants were harvested 24 h post-transfection and passed through a 0.45 μm filter. Cell-free viral culture supernatants were used to infect immortalized MEF cells in the presence of 8 μg/ml of polybrene. An additional round of infection was performed at 48 h and 72 h post-transfection. Infection was validated by GFP expression under fluorescent microscopy, and infected MEF cells were grown in 15-cm dishes before sorting at the same gate of fluorescence intensity using FACSAria cell sorter.

### Migration assay

MEF cells were grown and serum-starved for six h before plating on a ChemoTx chamber. Cells were detached with trypsin-EDTA and washed with serum-free DMEM. For the migration assay, the bottom side of the ChemoTx membrane was coated with type I collagen for 30 min and a total of $2 \times 10^4$ serum-starved cells in a 50 μl volume were placed on the top side of ChemoTx membrane. Migration was induced by placing the cells on an overlaid ChemoTx membrane on top of serum-free medium either in the absence or presence of chemotactic factors such as LPA and ascites from patients for 3 h. The ChemoTx membrane was

fixed with 4% paraformaldehyde, and non-migrated cells on the top side of membrane were removed by wiping with a cotton swab. The membrane was stained with DAPI, and migrated cells were counted with the fluorescence microscope at 10× magnification (Axiovert 200, Germany).

### Preparation of ascites from patients with ovarian cancer or liver cirrhosis

Ascites were obtained from four patients with stage III ovarian cancer (AOCP: range of age, 48-73 years) and four patients with liver cirrhosis (ALCP: range of age, 43-77 years) with the patients' consent as approved by the Institutional Review Board. Approximately 10 ml of ascitic fluid was collected and immediately centrifuged at 1000 × g for 20 min to remove cells. Each group of ascites was pooled together to eliminate individual variations and stored at -70°C until use.

### Immunoprecipitation and western blot analysis

Cells were lysed in lysis buffer containing HEPES-OH pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1 mM PMSF, 1.5 mM $Na_3VO_4$, 0.3% CHAPS, and protease inhibitor cocktail. Cleared cell extracts were mixed with 2 μg of the respective antibody (protein A agarose conjugated) and incubated for two h. Immunoprecipitates were washed with lysis buffer three times and sample buffer was added. Western blot analysis was performed as described previously (Bae *et al*., 2003).

### Statistical analysis

Results are expressed as the means ± S.D. of two independent experiments ($n$=3 for each experiment). When comparing two groups, an unpaired Student's $t$-test was used to address differences. $P$-values less than 0.05 were considered significant.

## Results

### LPA is major factor in AOCP that induces MEF cell migration

Ascites from malignant tumor-bearing patients such as ovarian cancer patients stimulate the migration of many types of cells. To ascertain that ascites from cancer patients contain inducing factor for MEF cell migration, we compared migration capability of ascites from ovarian cancer patients (AOCP) with that of liver cirrhosis patients (ALCP). As shown in Figure 1A, AOCP strongly stimulated MEF cell migration, however, ALCP showed only a marginal effect on the migration of MEF cells, indicating that AOCP contains inducing factor(s) for MEF cell migration. To identify responsible factor in AOCP that induces MEF cell migration, we have examined the effect of various receptor antagonists on the AOCP-induced MEF cell migration. Among the tested receptor antagonists, LPA receptor

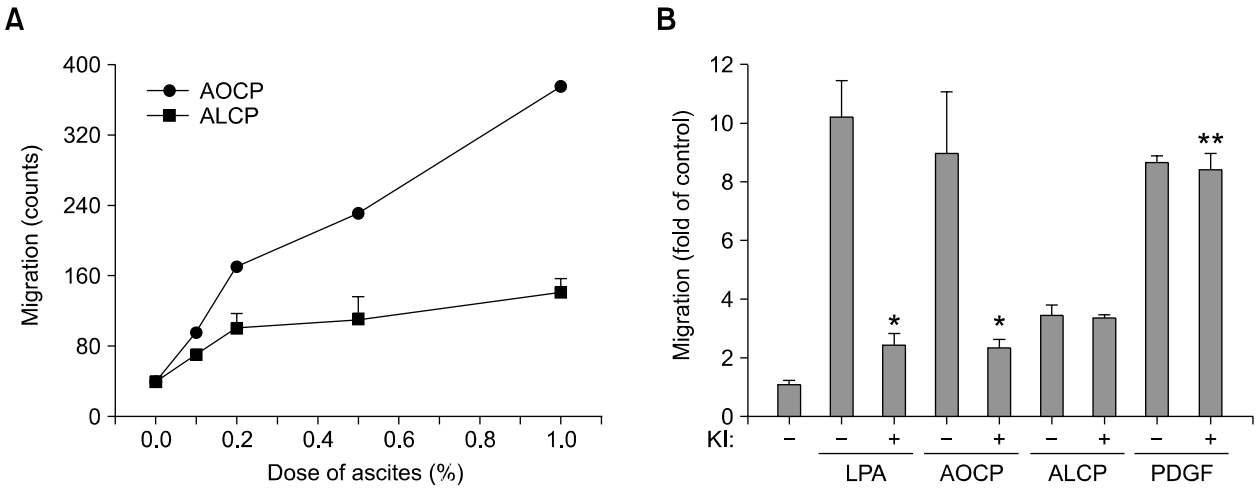


**Figure 1.** LPA is a major component of ascites from ovarian cancer patients (AOCP) that induces cell migration. (A) MEF cell migration was induced by the indicated dose of AOCP or ALCP (ascites from liver cirrhosis patients). (B) Migration of MEF cells was induced with LPA (5 μM), AOCP (1%), ALCP (1%), and PDGF (50 ng/ml) in either the presence or absence of LPA receptor antagonist (Ki: Ki16425, 10 μM). *Significantly different from cells stimulated with LPA or AOCP alone ($P < 0.05$). **Values were not statistically different from cells stimulated with PDGF alone ($P > 0.05$).

antagonist (Ki16425) completely blocked AOCP-induced cell migration as well as LPA-induced cell migration (Figure 1B). We also observed that Ki16425 did not affect PDGF-induced MEF cell migration. These results suggest that LPA is major responsible factor in AOCP that induce MEF cell migration.

### PI3K plays crucial roles in AOCP- and LPA-induced MEF cell migration

In order to investigate major signaling pathways that regulate LPA-induced MEF cell migration, we initially tried to block specific signaling pathways by using pharmacological inhibitors. As shown in Figure 2A, pretreatment of MEF cells with ERK (PD98059) and p38 MAPK inhibitor (SB303508) showed about 50% inhibition of LPA-induced MEF cell migration. Similarly, inhibition of ERK or p38 MPAK also resulted in 50% inhibition of AOCP-induced MEF cell migration (Figure 2B). However, inhibition of PI3K signaling pathway with LY294002 completely blocked both LPA- and AOCP-induced MEF cell migration (Figure 2). Therefore, ERK and p38 MPAK signaling pathways are also involved in LPA-induced cell migration but PI3K plays major role in downstream signaling pathway for LPA-induced MEF cell migration.

### Akt1 is required for LPA-induced MEF cell migration

Since PI3K signaling pathway plays pivotal roles in LPA-induced cell migration and Akt acts as major downstream effector of PI3K, we examined LPA-induced migration in cells lacking both *Akt1* and *Akt2*. As shown in Figure 3A, the double knockout (DKO) cells showed no protein expression for both Akt1 and Akt2 isoform. Deletion of both Akt1 and Akt2 resulted in complete loss of LPA-induced MEF cell migration (Figure 3B) demonstrating that either one or both of Akt isoform is required for LPA-induced cell migration. An isoform-specific function for Akt in LPA-induced MEF cell migration was validated by re-introduction of each Akt isoform in DKO cells. The expression level of each ectopic Akt1 and Akt2 was equivalent and was also about 100-fold higher than that of endogenous Akt in WT cells (Figure 3C). Under these experimental conditions, LPA-induced cell migration only occurred in the presence of Akt1 but not Akt2 (Figure 3D). Likewise, AOCP-induced DKO cell migration was restored by re-expression of Akt1 but not Akt2 (Figure 3E). Given these results, Akt1 is necessary for LPA-induced cell migration, and it is likely that Akt2 is dispensable for the LPA-induced MEF cell migration.

### LPA preferentially activates Akt1 rather than Akt2

Next, we examined the LPA-induced phosphorylation of Akt in MEF cells. As shown in Figure 4A, stimulation of cells with LPA rapidly induced the phosphorylation at $Ser^{473}$ of Akt. The saturating concentration of LPA for the induction of Akt phosphorylation was about 5 μM. Since Akt1 rather than Akt2 selectively mediated LPA-induced MEF cell migration (Figure 3), it is possible that Akt1 and Akt2 are differentially activated by LPA. However, it is very difficult to determine the activation of each isoform by LPA because MEF cells express all

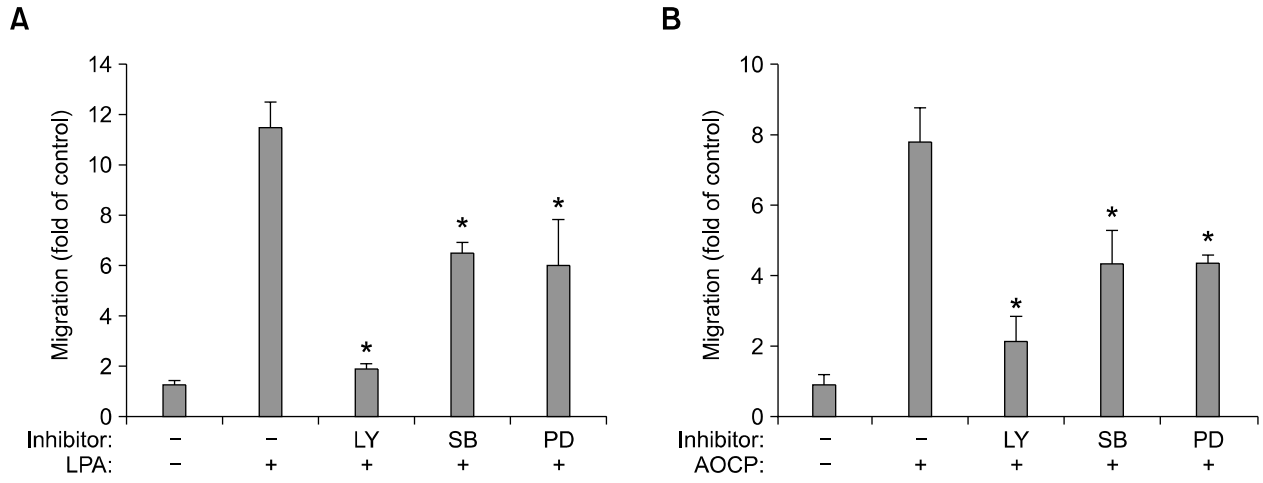


**Figure 2.** LPA-induced MEF cell migration is blocked by PI3K inhibitor. (A and B) MEF cell migration was induced by either LPA (5 μM) or AOCP (1%) in presence or absence of PI3K inhibitor (LY: LY294002, 10 μM), p38 MAPK inhibitor (SB: SB303580, 10 μM), and ERK inhibitor (PD: PD98059, 10 μM). *Significantly different from cells stimulated with LPA or AOCP alone ($P < 0.05$).

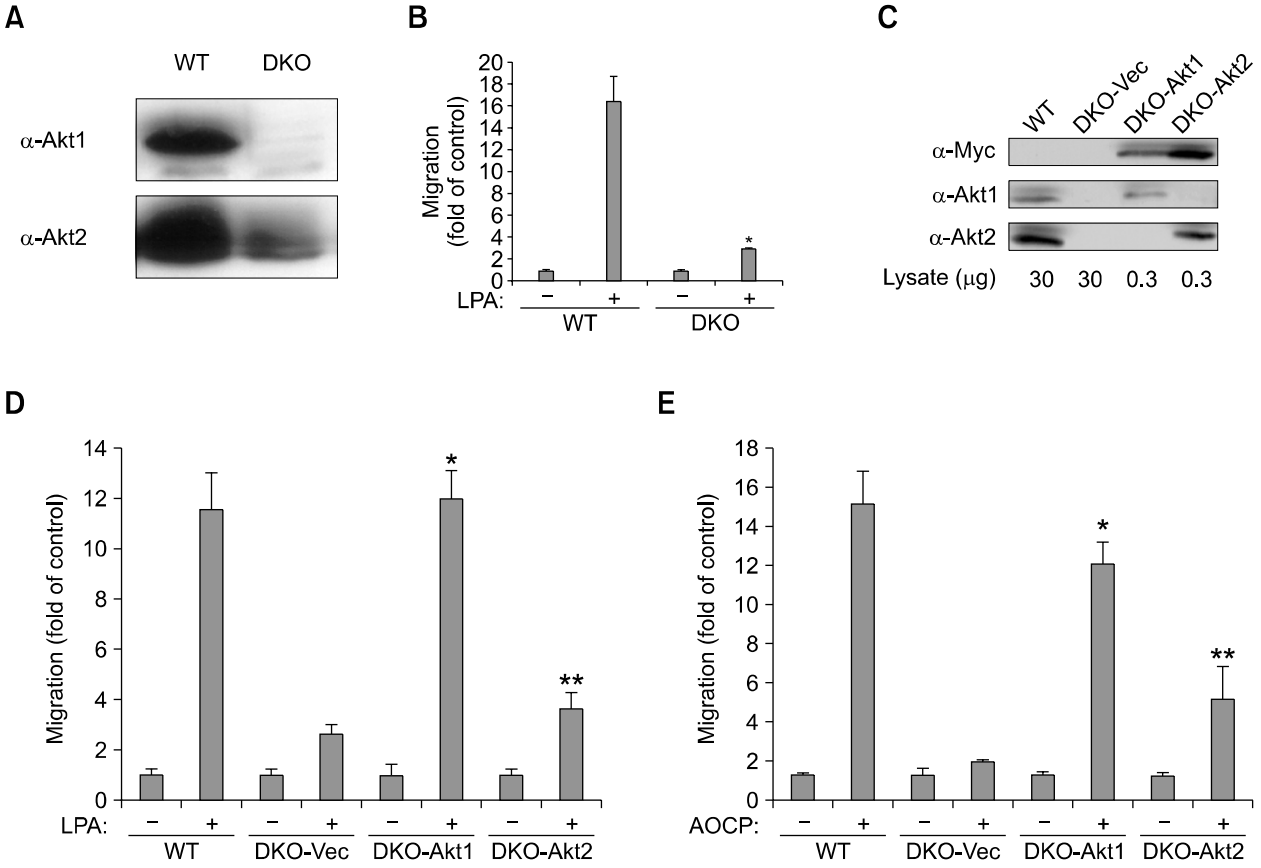


**Figure 3.** Akt1 is required for LPA-induced MEF cell migration. (A) Expression of each Akt isoform was ascertained by western blotting with indicated antibodies. (B) LPA-induced migration of established MEF cells was measured. *Significantly different from LPA-stimulated WT cells ($P < 0.05$). (C) Either Akt1 or Akt2 was re-introduced into *Akt1* and *Akt2* double deficient (DKO) cells. Ectopic expression of Akt1 or Akt2 was ascertained by western blotting with anti-myc antibody. Comparison of ectopic *versus* endogenous Akt levels was performed by western blotting with specific antibody against either Akt1 or Akt2. (D and E) LPA- or AOCP-induced migration in DKO cells after re-expression of either Akt1 or Akt2. *Significantly different from LPA- or AOCP-stimulated DKO-Vec cells ($P < 0.05$). **Values were not statistically different from LPA- or AOCP-stimulated DKO-Vec cells ($P > 0.05$).

three isoforms of Akt. Therefore, we have used DKO cells for the immunoprecipitation after re-expression of each isoform of Akt. As shown in Figure 4B, LPA strongly induced the phosphorylation of Akt1 whereas phosphorylation of Akt2 was relatively weak. Likewise, stimulation of MEF cells with AOCP resulted in the significant phosphorylation of Akt1 but relatively weak phosphorylation of Akt2. Therefore, it is likely that Akt1 is preferentially activated by LPA and plays pivotal roles in cell migration.

## Discussion

Plethora of reports suggests that ascites from cancer patients contain inducing factor for migration in many cell types. In fact, AOCP rather than ALCP strongly stimulated MEF cell migration (Figure 1A) indicating that ascites from cancer patients contain inducing factor for cell migration. LPA is one of the major lipid ligands that induce mesenchymal stem cell migration as well as ovarian cancer cell migration (Ren *et al*., 2006; Lee *et al*., 2007). Likewise, we also observed that AOCP-induced MEF cell migration was completely blocked by LPA receptor antagonist such as Ki16425 (Figure 1B). Considering that Ki16425 specifically inhibited LPA-induced cell migration but not PDGF-induced cell migration, it is reasonable that LPA is major component of AOCP that induces MEF cell migration.

It has been reported that LPA activates many signaling pathways including the Ras/MEKK/MAPK, p38 MAPK, and JNK pathways (Bian *et al*., 2004; Malchinkhuu *et al*., 2005). It also has been reported that LPA stimulates DNA synthesis *via* activation of PI3K (Koh *et al*., 1998). In correlation

with this, pharmacological inhibitions of ERK and p38 MAPK resulted in the attenuation of LPA-induced MEF cells migration but lesser extent than PI3K inhibitor (Figure 2). However, inhibition of PI3K completely blocked LPA-induced MEF cell migration, indicating that activation of PI3K is crucial event for LPA-induced MEF cell migration. Currently, the mechanism by which LPA activates PI3K could be explained by two modes of activation. First, transactivation of EGFR represents cross-talk between receptor tyrosine kinases and GPCR. For example, stimulation of squamous cell carcinoma cell lines with GPCR agonists evokes the tyrosine phosphorylation of EGFR and activation of PI3K (Gschwind *et al*., 2002; Shah *et al*., 2006). Since the inhibition of metalloprotease inhibitor blocks GPCR agonists- induced activation of EGFR, the communication between GPCR and EGFR signaling systems involves cell surface proteolysis of EGF-like precursors (Gschwind *et al*., 2003). Currently, how GPCR- induced activation of trimeric G proteins leads to the activation of metalloprotease is still unknown. Second, LPA could directly activate class II PI3K and generate phosphatidylinositol 3'-phosphate (Maffucci *et al*., 2005). However, it is notable that the lipid product of class II PI3K is not able to activate downstream Akt signaling pathway.

Although all Akt isoforms share about 85% amino acid sequence homology, different roles for each isoform in many physiological responses have been elucidated by targeted disruption of each *Akt* gene in mice (Cho *et al*., 2001a, b; Ackah *et al*., 2005; Fernandez-Hernando *et al*., 2007). Distinctive roles of Akt isoform in many cellular physiologies also have been reported. For example, cells lacking Akt1 show impairment of adipogenesis (Baudry *et al*., 2006). On the other hand, GLUT4 translocation and glucose uptake is uniquely regulated by Akt2 (Bae *et al*., 2003). In correlate with this, our results also showed that LPA-induced MEF migration was distinctively regulated by Akt isoform. For example, LPA-induced cell migration was abrogated in cells lacking *Akt1*, and most importantly, impairment of LPA-induced migration of DKO cells was restored by Akt1 but not Akt2 (Figure 3). Since 100-fold over-expression of Akt2 did not rescue impairment of LPA-induced DKO cell migration, there might be differences in intrinsic properties of Akt isoform during the regulation of LPA-induced migration. It is also noteworthy that Akt2 is required for the GLUT4 translocation and glucose uptake in the same MEF cells that we have used in this study (Bae *et al*., 2003).

There is controversy surrounding the idea of LPA-induced activation of Akt, which might be due to differences in cellular context. For example, LPA does not activate Akt in cancer cells such as SKOV-3 and HeLa cells but shows high basal activity (Maffucci *et al*., 2005). However, normal fibroblasts such as MEF cells show LPA-induced activation of Akt, as cells lacking the $LPA_1$ and $LPA_2$ receptors show a defect in Akt activation (Contos *et al*., 2002). Likewise, we have observed that LPA stimulates phosphorylation at $Ser^{473}$ of Akt in a time- and dose-dependent manner (Figure 4). Most importantly, LPA preferentially induced the phosphorylation at $Ser^{473}$ of Akt1 implicating that Akt1 rather than Akt2 plays major role in LPA-induced cell migration. In correlation with this interpretation, we also have observed that LPA

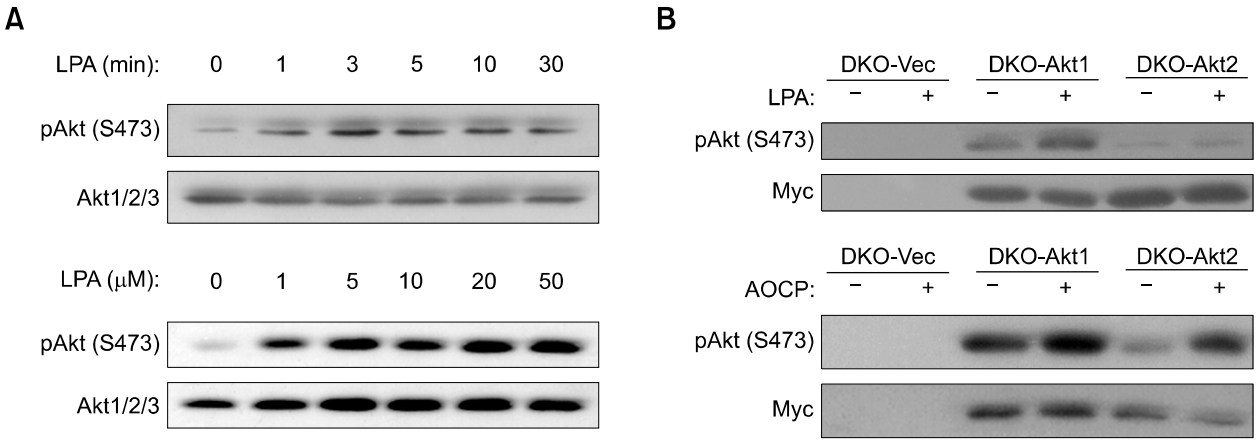


**Figure 4.** Akt1 is selectively activated by LPA stimulation. (A) Cells were either stimulated with LPA (5 μM) for indicated time or stimulated with indicated dose of LPA for 5 min, and LPA-induced phosphorylation at $Ser^{473}$ of Akt and total Akt level was ascertained by western blotting. (B) Cells were stimulated with either LPA (5 μM) or AOCP (1%) for 5 min and cell extracts were immunoprecipitated with anti-myc antibody. Total Akt and phosphorylated Akt were ascertained by western blotting with anti-myc and anti-phospho-Akt (Ser473) antibodies, respectively.

requires Akt1 during the migration (Figure 3). The mechanism of how the LPA receptor signaling pathway is coupled to the activation of Akt1 is still ambiguous. Recent evidence suggests that P-Rex1 (Rac guanine nucleotide exchange factor), which is activated by both PI3K and the $G_{\beta\gamma}$ subunit of trimeric G proteins, regulates cell migration through the molecular complex formation with mammalian target of rapamycin (mTOR) (Hernandez-Negrete *et al*., 2007). mTOR complex is also known to play essential roles in migration as well as the activation of Akt (Sarbassov *et al*., 2004, 2005). We also have discovered that linker region of Akt1 plays essential roles in PDGFR-induced cell migration by targeting Akt1 to the membrane ruffles (data not shown). Therefore, it is likely that differences in intrinsic properties of each Akt kinase may establish different roles of Akt isoform in many cellular physiologies. In this regard, examination on the molecular complex formation of each Akt isoform may be necessary to delineate signaling specificity during LPA-induced cell migration.

In summary, we have demonstrated that LPA is a major component of ascites from ovarian cancer patients that induces cell migration through the PI3K signaling pathway. Among the Akt isoforms, Akt1 was selectively activated and played essential roles during LPA-induced cell migration. Our studies suggest that each Akt isoform may have different intrinsic properties and analysis of their functional domains will shed more light on the Akt-mediated signaling pathway of cell migration.

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