

## Lysophosphatidic Acid Stimulates Urokinase Receptor (uPAR/CD87) in Ovarian Epithelial Cancer Cells

JASON LIZALEK, TIM MCKENNA, KYLE HUEGEL, SYDNEY MARSH, ALEXANDRA CAROLAN, AMY KOBLISKA, EMILY HEYING, NICOLE GARDNER, GEOFF MILLER, AARON KOTECKI, MEGHAN HENNINGSEN, ANASTASIA LUNDT, JANEAN FARLEY and SHAWN M. ELLERBROEK

*Department of Biochemistry, Chemistry, and Engineering Sciences, Wartburg College, Waverly, IA, U.S.A.*

**Abstract.** *Background/Aim:* Lysophosphatidic acid (LPA) is a bioactive lipid positively linked with ovarian cancer progression. The multi-functional urokinase receptor (uPAR), a cell-surface glycoprotein, binds and facilitates activation of uPA and laterally regulates integrin and tyrosine kinase receptor activities in promotion of cell migration and invasion. We hypothesized that LPA stimulates uPAR expression and activity in ovarian epithelial cancer cells. *Materials and Methods:* Ovarian epithelial cancer cell lines OVCA 429 and OVCA 433 were stimulated with LPA and examined for uPAR mRNA expression and protein localization. uPA binding to OVCA plasma membranes was measured through enzymatic analysis of affinity-isolated cell-surface proteins. *Results:* LPA drove cell-surface uPAR aggregation and mRNA expression concomitant with increased cell-surface binding of uPA. Both control and LPA-stimulated uPAR expression and uPA cell-surface association involved phosphatidylinositol 3-kinase, but not p38 or p42 mitogen-activated protein kinase, signaling. *Conclusion:* These data provide mechanistic insight into ovarian epithelial cancer cell progression by demonstrating that LPA drives uPAR expression and uPA binding.

Lysophosphatidic acid (LPA) is a bioactive lipid positively linked with ovarian cancer progression (1). In accordance with this, the concentration of LPA is increased in ascitic fluid and malignant effusions of patients with ovarian cancer (2, 3). Similar elevated levels of LPA have been found in patients with other types of gynecological cancers, including primary peritoneal, endometrial, and cervical malignancies (4). Several plasma membrane receptors for LPA have been identified, including the endothelial differentiation gene

receptor-2 (EDG2)/LPA<sub>1</sub>, EDG4/LPA<sub>2</sub> receptor, and the EDG7/LPA<sub>3</sub> receptors (5, 6). Notably, ovarian epithelial cancer cell growth and invasion *in vitro* and *in situ* has been reported to require expression of LPA receptors (7).

Urokinase-type plasminogen activator (uPA) is a serine protease that once bound to its receptor (uPAR/CD87) can catalyze the activation of plasminogen into the active protease plasmin on the surface of cells, which in turn contributes to a number of proteolysis-dependent events, including cellular metastasis (8, 9). The activity and expression of uPA and its multi-function receptor uPAR have both been identified as contributors to ovarian cancer metastasis (10, 11). Markedly, direct inhibition of either uPA or uPAR protein function reduces ovarian cancer tumorigenicity (12, 13). LPA is an established stimulator of uPA expression and activity in ovarian cancer cells, suggesting a potential mechanistic basis of LPA-driven metastatic behavior (14). Further, treatment of ovarian cancer cell lines with ascitic fluid has been shown to stimulate uPAR expression, however the mediators of this ascitic effect have not been identified (15). As LPA promoted uPAR mRNA expression in a gastric cancer cell line (16), we hypothesized that LPA would stimulate uPAR expression and activity in two invasive ovarian epithelial cancer cell lines, OVCA 429 and OVCA 433.

### Materials and Methods

*Cell culture and LPA treatment.* OVCA 429 and OVCA 433 ovarian epithelial cancer cell lines and human plasminogen were provided by Dr. Sharon Stack (Harper Cancer Research Institute, South Bend, IN, USA). Cell cultures were maintained on 10-cm dishes in the presence of minimum essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Biowhittaker, Walkersville, MD, USA). LPA (18:1) was purchased from Avanti Polar Lipids (Alabaster, AB, USA), dissolved in chloroform, evaporated, and resuspended in phosphate buffered saline (PBS) containing 1% delipidated bovine serum albumin (BSA) (#A1595; Sigma-Aldrich, St. Louis, MO, USA). Cells were serum starved for 3 h prior to administration of fresh serum media containing 80  $\mu$ M LPA. Control

*Correspondence to:* Shawn Ellerbroek, Ph.D., 100 Wartburg Blvd, Department of BCES, Waverly, IA 50677, U.S.A. Tel: +1 9199395845, e-mail: shawn.ellerbroek@wartburg.edu

*Key Words:* Lysophosphatidic acid, uPAR, ovarian, uPA, PI3K.

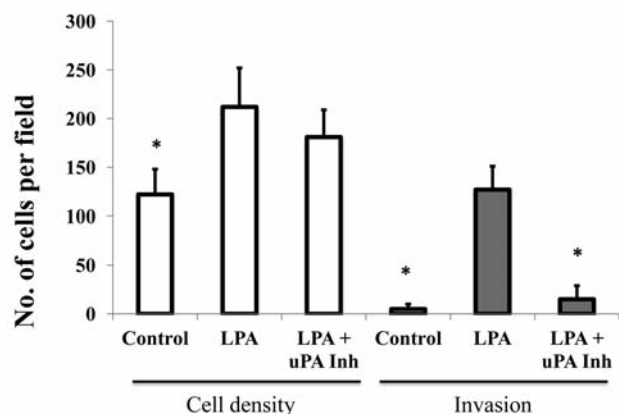


Figure 1. *Lysophosphatidic acid (LPA) effectively stimulates an invasive profile in ovarian cancer cells. LPA stimulation of ovarian cancer proliferation and invasion requires urokinase-type plasminogen activator (uPA) activity (OVCA 433). \*Difference in cell density compared to the control was statistically significant with a p-value of  $5.8 \times 10^{-8}$ . LPA-driven invasion was significantly greater compared to both control and LPA/uPA inhibitor-treated cells, with p-values of  $7.5 \times 10^{-18}$  and  $2.3 \times 10^{-15}$  respectively. Statistics were calculated using two-tailed, unpaired, equal variance t-tests with 14 degrees of freedom.*

cells received the equivalent concentration of delipidated BSA (0.04%). Phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (10  $\mu$ M) (Calbiochem, Temecula, CA, USA) or dimethyl sulfoxide (DMSO) vehicle control was administered in fresh serum-free media 1-h prior to LPA-stimulation. Upon stimulation, fresh LY294002 inhibitor or vehicle control was mixed in with the new control or LPA-containing serum-free media administered to cells.

**Matrigel invasion assay.** OVCA cells stimulated as described above were plated ( $5 \times 10^5 \text{ ml}^{-1}$ ) in triplicate into Matrigel-coated (11  $\mu$ g per filter) invasion chambers (8 micron, BD#354578) in the presence or absence of 10  $\mu$ g  $\text{ml}^{-1}$  anti-catalytic uPA antibody (#394; American Diagnostica, Lexington, MA, USA) or IgG control (Sigma-Aldrich). After 48 hours, cell invasion was stopped with a Diff-Quick Kit (Fisher Scientific, Pittsburgh, PA, USA) and invading cells counted through 5 random  $\times 10$  objective lens fields per filter. For density control, cells were plated onto tissue culture plastic, incubated for 48 hours, and counted as described above.

**Immunofluorescence.** OVCA cells were grown overnight on ethanol-washed glass coverslips in the presence of serum prior to serum starvation and LPA stimulation as described above. Cells were fixed for 10 min in 3.7% formaldehyde in PBS. PBS-washed cells were incubated with 1  $\mu$ g  $\text{ml}^{-1}$  PBS of either anti-E-cadherin monoclonal antibody (#HECD-1; Invitrogen) or anti-uPAR monoclonal antibody (#3936; American Diagnostica). Immunofluorescence staining was visualized using an Alexa Fluor 488 goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Images were obtained on an Olympus spinning disc confocal microscope using a CoolSNAP E2 CCD camera (Photometrics, Tucson, AZ, USA) and Metamorph Image software (Universal Imaging Corporation, Bedford Hills, NY, USA). Permeabilization

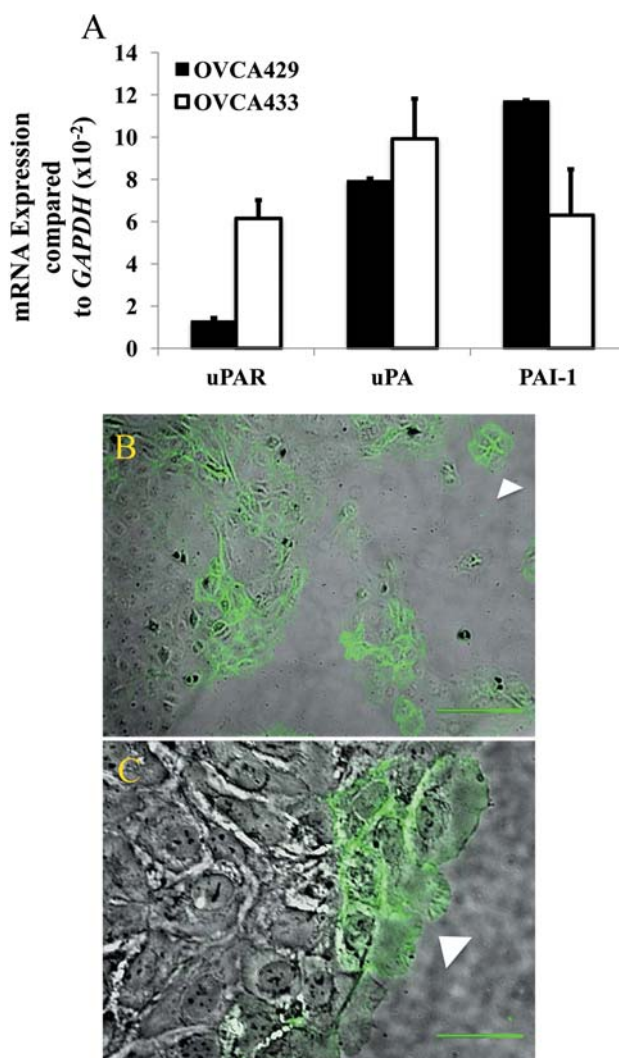


Figure 2. *OVCA cells exhibit heterogeneous urokinase-type plasminogen activator receptor (uPAR) expression. A: OVCA 433 cells have higher basal expression of uPAR mRNA than OVCA 429 cells. B-C: uPAR protein expression in OVCA 433 cells is heterogeneous, highest at peripheral island cells, and localized to membrane protrusions and cell borders throughout (white arrowheads). Bar=205  $\mu$ m (B) and 30  $\mu$ m (C). Overlay: uPAR (green) over bright-field image.*

of cells with Triton X-100 (0.5%) or pre-treatment with phosphatidylinositol phospholipase-C (Sigma-Aldrich) altered or removed uPAR staining, validating antibody reactivity to the cell-surface glycoprotein (Supplemental Figure 1).

**uPA Activity.** uPA activity in conditioned media and cell-surface protein samples was measured in triplicate in a 96-well plate using a coupled assay that monitors human plasmin generation and subsequent cleavage of a valine-leucine-lysine-para-nitroanilide (Sigma-Aldrich) substrate (17). Absorbance at 405 nm was monitored every 30 seconds for one hour to produce velocity data.

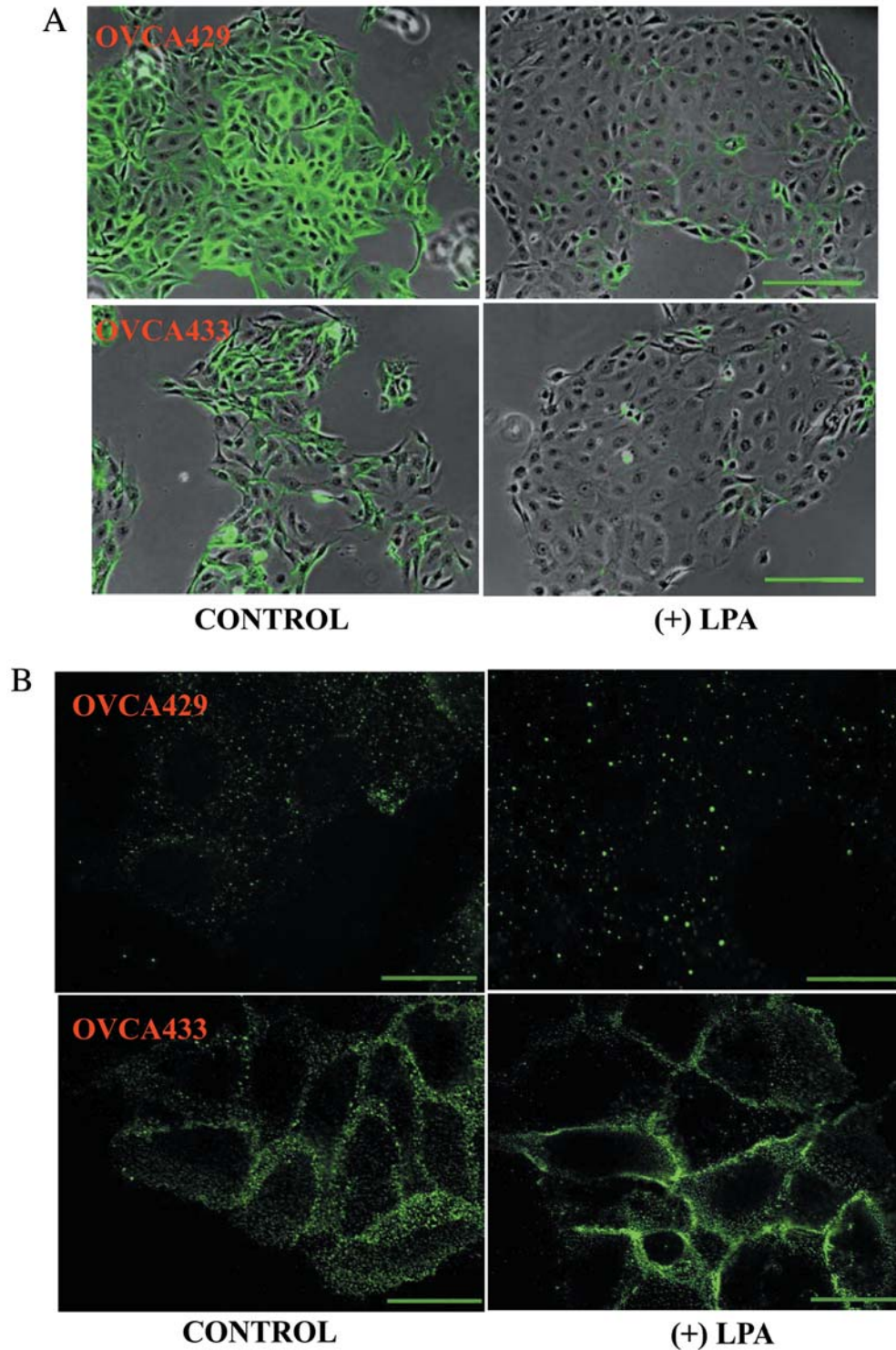


Figure 3. Effects of lysophosphatidic acid (LPA) on cell morphology and urokinase-type plasminogen activator receptor (uPAR) localization. A: LPA effectively promotes E-cadherin dissolution at 2 h (E-cadherin green, overlaid with differential interference contrast image). E-Cadherin mRNA levels were not changed (N=3). LPA-stimulated OVCA 429 and OVCA 433 cells appear phenotypically similar, with increased cell spreading. Bar=205  $\mu$ m. B: At high magnification, OVCA 429 cells have low speckled basal expression of uPAR that becomes brighter and more punctate upon LPA stimulation. The cell border-associated uPAR staining on OVCA 433 cells became more focused or compressed upon LPA stimulation. Thus, while these two different ovarian epithelial cancer cell lines exhibited unique uPAR protein expression, both exhibited uPAR aggregation following LPA stimulation. Bar=30  $\mu$ m. Overlay: uPAR (green) over bright-field image.

**Analysis of mRNA transcript.** Control and LPA-stimulated cells were lysed with TRIzol reagent (Ambion, Carlsbad, CA, USA), mRNA isolated, and cDNA produced using reverse transcriptase (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturers' protocols. Transcript levels were quantified using TaqMan Real-Time PCR master mix (Applied Biosystems) and a StepOnePlus Real-Time PCR system. All values were normalized to *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* transcript levels. Primers used were Hs01547054\_M1 (*uPA*), Hs01126607\_G1 (*plasminogen activator inhibitor-1 (PAI-1)*), Hs00958880 (*uPAR*) and Hs03929097\_G1 (*GAPDH*) from Applied Biosystems.

**Isolation of biotinylated cell surface proteins.** Control and LPA-stimulated cells on 10 cm dishes (approximately 70-80% confluent) were washed twice with volumes of cold cell-culture grade PBS (pH 7.4) (Invitrogen). To biotinylate surface proteins, cells were incubated with 3 ml of cold sulfo-*N*-hydroxysuccinimide (NHS)-biotin (Pierce, Carlsbad, CA, USA)/PBS (pH 7.4) at a concentration of 100 µg ml<sup>-1</sup> and gently rocked at 4°C for 15 minutes. Control (no biotin) cells received only PBS. After rocking, dishes were washed twice with PBS and then incubated another 15 minutes with 5 ml of cold 100 mM glycine/PBS solution (pH 8.0) to neutralize unreacted biotin. After three washes with cold PBS, each cell plate was lysed with 1 ml of cold lysis buffer pH 8.0 (50 mM sodium phosphate, 300 mM NaCl, 1% Triton X-100) with 1% protease inhibitor cocktail (Sigma-Aldrich). Clarified lysate (13,000 × g for 15 minutes) was quantified using a BCA protein reagent assay (Pierce). Cell lysates were diluted to 750 µg ml<sup>-1</sup> and then a 1ml volume of each lysate incubated end-over-end with 50 µl of immobilized monomeric avidin at 4°C for 60 minutes. Each sample was then washed five times with 1 ml with cold PBS and isolated biotinylated proteins eluted through the addition of 10 mM D-Biotin (Sigma). Eluted cell-surface proteins were analyzed for uPA activity as described below. High-speed (100,000 × g) isolation of plasma membranes confirmed the presence of biotin-tagged protein only in the P100 (membrane pellet) fraction (Supplemental Figure 2). Successful isolation of cell surface uPA activity was sensitive to a brief acid-washing pre-treatment of cells before biotinylation, indicating biotin-captured isolates reflect cell surface-bound uPA (Supplemental Figure 3).

## Results

LPA-stimulated invasion required extracellular uPA activity, confirming that this serine protease is an important participant in the lipid-induced *in vitro* invasion of OVCA cells (Figure 1). To understand the impact of LPA on the uPA/uPAR system in these cells, basal transcript levels for *uPA*, *uPAR*, and *PAII* were first established. OVCA 433 cells retain much lower basal expression of *PAII* mRNA while demonstrating higher transcript expression levels of both *uPAR* and *uPA* than OVCA 429 cells (Figure 2A). In accordance, uPAR signal was more robust in OVCA 433 cells than OVCA 429 cells and localized to the periphery of cell islands near sites of membrane ruffling (Figures 2B and C).

After a two-hour LPA stimulation, both OVCA 429 and OVCA 433 cells exhibited similar E-cadherin dissolution and flattened cellular morphology, consistent with the adoption of an invasive phenotype (Figure 3A). By immunofluorescence,

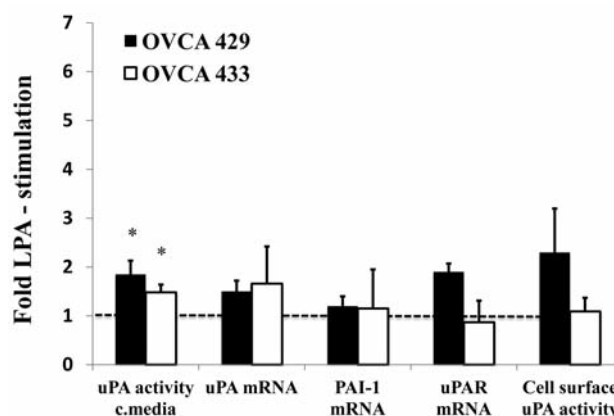


Figure 4. Short-term lysophosphatidic acid (LPA) effects on mRNA expression. Serum-starved cells were stimulated for 2 h with 80 µM LPA prior to analysis. urokinase-type plasminogen activator (uPA) activity in conditioned media was slightly, but significantly up-regulated in both cell lines post-LPA stimulation (Mann Whitney U, \* $p=0.005$ ,  $z=-2.8$ ,  $U=0$ ). uPA mRNA levels were also higher, but by less than two-fold. urokinase-type plasminogen activator receptor (uPAR) mRNA was approximately two-fold higher in OVCA 429 cells, whereas OVCA 433 uPAR mRNA was unchanged ( $N=5$ ). In accordance, the amount of plasma membrane-associated uPA was stimulated by LPA in OVCA 429, but not OVCA 433 cells ( $N=4$ , two experiments).

both cells exhibited clustering of uPAR on the cell surface in response to LPA, with OVCA 429 cells exhibiting a weaker punctate distribution and OVCA 433 cells a brighter and tighter distribution at cell junctions (Figure 3B).

Both cell lines exhibit only a modest change in uPA activity in conditioned media following 2-h LPA stimulation, with neither *uPA* nor *PAII* transcripts being significantly altered (Figure 4). OVCA 429 cells, but not OVCA 433 cells, exhibited a two-fold increase in uPAR expression. In accordance with this increase in uPA receptor levels, an approximately equal change in cell-surface associated uPA activity was observed for OVCA 429 cells. Taken together, these data suggest different profiles exist for both basal and LPA-stimulated expression of *uPAR* mRNA between the two OVCA cell lines, although both presented cell-surface uPAR aggregation following LPA stimulation.

In order to assess longer-term effects of LPA stimulation, both cell lines were stimulated for either 24 or 36 h with LPA. Thirty-six hours post LPA stimulation, OVCA 433, but not OVCA 429 cells, exhibited a clear increase in uPA activity in the conditioned media (Figure 5A), although *uPA* mRNA for both cell lines was increased less than two-fold at 24 h. OVCA 429 cells exhibited a 4±2-fold increase in *uPAR* mRNA expression, whereas OVCA 433 cells, which had a much higher basal expression of *uPAR* mRNA and protein immunostaining, were relatively unchanged in their *uPAR* mRNA expression. Once again, an increase in *uPAR*

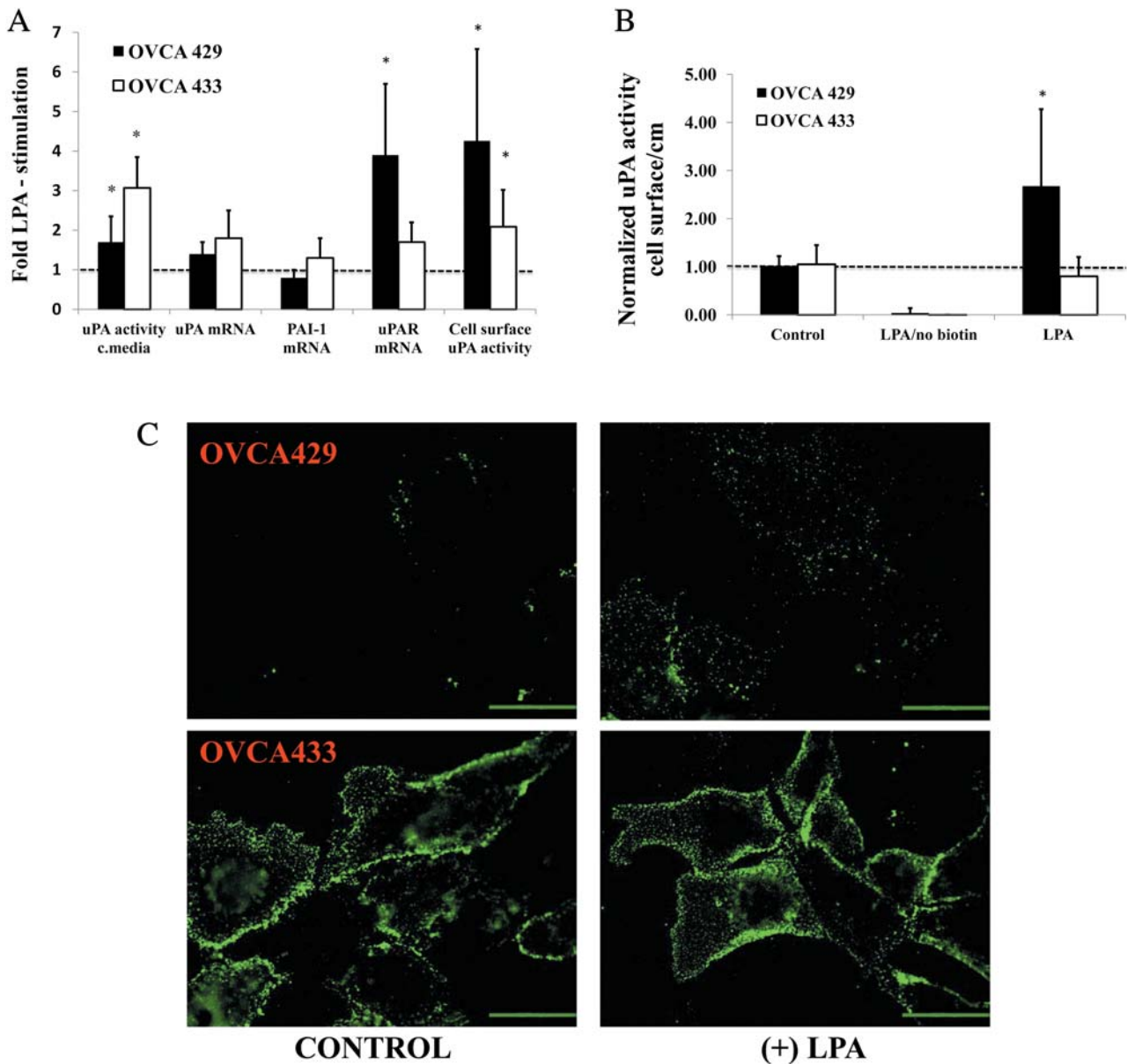


Figure 5. Long-term lysophosphatidic acid (LPA) effects on the urokinase-type plasminogen activator (uPA) system. A: LPA-stimulated uPA activity of both OVCA cell lines remained significantly higher in the conditioned media after 36 h compared to control (OVCA 429,  $*p=0.0035$ ,  $N=16$ ,  $Z=-2.923$ ,  $U=27$  and OVCA 433,  $p=8 \times 10^{-5}$ ,  $N=14$ ,  $z=-3.9399$ ,  $U=0$ ), although uPA mRNA changes were modest. LPA stimulation of uPAR mRNA in OVCA 429 cells at 24 hours ( $p=0.0098$ ,  $N=12$ ,  $Z=-2.5825$ ,  $U=5$ ) and cell surface-associated uPA activity at 36 h ( $p=0.0001$ ,  $N=11$ , five experiments,  $z=-3.8378$ ,  $U=0$ ) were also significantly higher than those of the control. LPA stimulation of OVCA 433 uPAR mRNA at 24 h was not significant ( $p=0.11$ ,  $N=9$ ,  $z=-1.587$ ,  $U=19$ ), although cell surface binding was statistically different compared to controls ( $p=0.001$ ,  $N=11$ , five experiments,  $z=-3.2833$ ,  $U=10$ ). (B) The ratio of uPA activity on the cell surface to that of conditioned media is significantly higher for LPA-stimulated OVCA 429 cells compared to control ( $p=0.015$ ,  $N=13$ ,  $Z=-2.403$ ,  $U=37$ ), but not for OVCA 433 cells ( $p=0.2113$ ,  $N=11$ ,  $z=1.246$ ,  $U=41$ ). (C) Consistent with these data, OVCA 429 cells displayed higher uPAR immunofluorescence at 24 h, whereas cellular uPAR staining in OVCA 433 cells remained relatively unchanged. Bar=30 μm.

mRNA levels resulted in an approximately equal rise in cell surface-associated uPA activity (Figure 5A). The increased binding of uPA to the cell surface appears dependent on amplified receptor activity and independent of uPA

concentration in the conditioned media, as the ratio of uPA activity at the cell surface compared to conditioned media was significantly stimulated in OVCA 429, but not OVCA 433 cells (Figure 5B). In support of these findings, increased

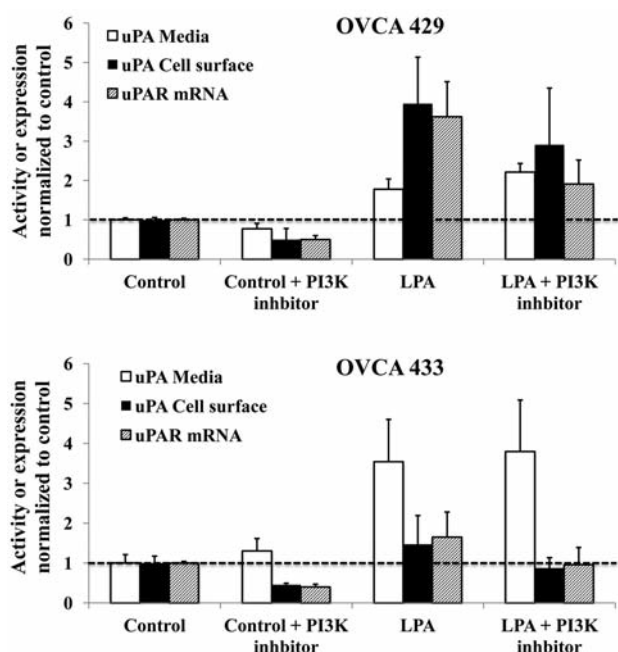


Figure 6. Contribution of phosphatidylinositol 3-kinase (PI3K) signaling to cell-surface urokinase-type plasminogen activator (uPA) association. Inhibition of PI3K signaling (10  $\mu$ M LY294002), had a nominal to slightly positive effect on uPA activity in the conditioned media from either OVCA 429 or OVCA 433 cells under both control and lysophosphatidic acid (LPA)-stimulated states (36 h). At the same time, PI3K inhibition reduced cell-surface association of uPA in both OVCA 429 and OVCA 433 cells, regardless of LPA stimulation (N=4, two experiments). In accordance with this, the decrease in surface-associated uPA activity was reflected by a reduction in urokinase-type plasminogen activator receptor (uPAR) mRNA levels (N=6, two experiments).

uPAR staining of OVCA 429 cells was evident after long-term LPA stimulation (Figure 5C).

Aberrant PI3K activity has been linked with ovarian cancer progression and functionally with uPAR signaling (18, 19). Furthermore, LPA-driven migration of fibroblasts requires PI3K activity (20). We, therefore, analyzed whether PI3K contributed to LPA-stimulated uPAR expression and binding of uPA to the cell surface. Treatment of cells with the PI3K inhibitor LY294002 had no to modest effects on the release of uPA into conditioned media by either OVCA 429 or OVCA 433 cells (Figure 6). At the same time, PI3K inhibition caused a significant reduction (30-50%) in both uPAR mRNA levels and cell surface-associated uPA activity in both cell lines under all conditions. However, as the fold-stimulation in uPAR mRNA and cell surface-associated uPA activity by LPA were maintained or slightly augmented under conditions of PI3K inhibition, we conclude that PI3K signaling is not involved in LPA-stimulated uPAR expression in these cells and downstream PI3K signaling appears to govern core expression of this receptor.

## Discussion

Ovarian cancer, the leading cause of death from gynecological malignancies in the United States, has been estimated to claim the lives of over 14,000 women annually (21). As early detection is difficult due to the ambiguity of symptoms (22), mechanistic identification of ovarian cancer progression and metastasis is critical for both clarifying early detection targets and development of therapeutic treatments.

We found that LPA-stimulation promoted uPAR expression and activity to differing extents in the studied cell lines, OVCA 429 and OVCA 433. Similar to MDA-MB-231 breast cancer cells (23), uPAR was found localized to the leading edges and membrane ruffles of OVCA 433 cell cultures. In contrast, uPAR was more punctate in distribution on the surface of OVCA 429 cells. After two hours of LPA stimulation, cell-surface uPAR was found to aggregate in both cell types. Interestingly, Ahn *et al.* have recently reported that uPAR laterally associates with  $\alpha$ V $\beta$ 6 integrins in OVCA 429 cells (24, 25); it is possible that LPA drives uPAR/ $\alpha$ V $\beta$ 6 association, as indicated by the punctate nature of the staining. On the other hand, LPA focused uPAR staining to the lateral edges of OVCA 433 cells.

As uPA activity has been reported to drive E-cadherin turnover in OVCA 429 cells (26) and both OVCA 429 and OVCA 433 cells exhibit junctional dissolution (Figure 2A), we examined whether uPAR was required for E-cadherin dissolution. Although we did not block uPAR aggregation, we did find that inhibiting over 90% of uPA activity in the media with the same function-blocking antibody that impeded matrigel invasion had no effect on the ability of LPA to drive E-cadherin turnover in OVCA 433 cells. LPA was also found to drive matrix metalloproteinase 9 (MMP9)-mediated proteolysis of E-cadherin in this cell line (27). Thus, it appears that both uPA and MMP9 proteases may contribute to regulation of E-cadherin shedding downstream of LPA signaling. Altogether, the heterogeneity of uPAR staining between these two cell lines is indicative of both the multi-functionality of the uPAR glycoprotein and the potential for it to assume different contributory roles in ovarian epithelial cancer.

After 24-h of LPA stimulation, there was a notable increase in uPAR expression in OVCA 429, but less so in OVCA 433 cells. By normalizing to GAPDH, it appears that though uPAR expression is less inducible in OVCA 433 cells, they express higher basal amounts of uPAR mRNA than OVCA 429 cells. This conclusion was strongly supported by the intensity of the uPAR immunofluorescence staining between the two cell lines. Hence, the heterogeneity between these two ovarian epithelial cell lines extends to LPA regulation of uPAR gene expression.

Both cell lines did exhibit increases in cell-surface uPA binding that approximated the observed changes in their *uPAR* mRNA expression, suggesting that stimulation of *uPAR* transcript levels led to increased cell surface uPAR expression (Figure 3). Neither p42/44 MAPK (PD98059, 10  $\mu$ M), p38 MAPK (SB202190, 10  $\mu$ M) nor PI3K (LY294002, 10  $\mu$ M) inhibitors effectively blocked LPA stimulation of uPAR expression and uPA cell-surface binding, although the PI3K inhibitor did reduce up to 50% of both basal and LPA-stimulated uPA binding and *uPAR* mRNA expression in both cell lines (Figure 6). As PI3K activity has been reported to be dispensable for clathrin/low density lipoprotein receptor-related protein 1-mediated uPAR recycling from the cell surface (28), and PI3K inhibition resulted in an equivalent reduction in *uPAR* mRNA as with cell-surface binding (Figure 6), the contribution of PI3K signaling to uPAR regulation appears primarily at the level of gene expression.

In conclusion, we showed that LPA stimulation of ovarian cancer cells induces uPAR expression followed by a proportional increase in cell surface binding of uPA. The ability of LPA to drive uPAR expression and uPA cell-surface recruitment adds mechanistic insight into ovarian cancer progression, while our finding of heterogeneity on uPAR expression, localization, and induction between the two OVCA cell lines underscores the multifaceted nature of LPA–uPAR-driven ovarian cancer metastasis.

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