Lysophosphatidic Acid Down-Regulates Stress Fibers and Up-Regulates Pro–Matrix Metalloproteinase-2 Activation in Ovarian Cancer Cells

Thuy-Vy Do,1 Jay C. Symowicz,1 David M. Berman,3 Lance A. Liotta,3 Emanuel F. Petricoin III,4 M. Sharon Stack,2 and David A. Fishman5

Departments of 1Obstetrics and Gynecology and 2Cell and Molecular Biology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois; 3Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland; 4Office of the Director, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, Maryland; and 5Department of Obstetrics and Gynecology, Gynecologic Oncology, New York, New York

Abstract
Epithelial ovarian cancer (EOC) is asymptomatic at early stages and is often diagnosed late when tumor cells are highly metastatic. Lysophosphatidic acid (LPA) has been implicated in ovarian oncogenesis as levels of this lipid are elevated in patient ascites and plasma. Because the underlying mechanism governing LPA regulation of matrix metalloproteinase-2 (MMP-2) activation remains undefined, we investigated the relationship between LPA-induced changes in actin microfilament organization and MMP-2 enzymatic activity. We report that when cells were cultured at a high density, LPA mediated stress fiber and focal adhesion disassembly and significantly repressed RhoA activity in EOC cells. Inhibition of Rho-kinase/ROCK enhanced both LPA-stimulated loss of stress fibers and pro-MMP-2 activation. In contrast, expression of the constitutively active RhoA(G14V) mutant diminished LPA-induced pro-MMP-2 activation. LPA had no effects on membrane type 1–MMP or tissue inhibitor of metalloproteinase-2 expression, but up-regulated MMP-2 levels, contributing to the induction of MMP-2 activation. Interestingly, when cells were cultured at a low density, stress fibers were present after LPA stimulation, and ROCK activity was required for EOC cell migration. Collectively, these results were consistent with a model in which LPA stimulates the metastatic dissemination of EOC cells by initiating loss of adhesion and metalloproteinase activation. (Mol Cancer Res 2007;5(2):121–31)

Introduction
Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy among women in the Western world. More than two-thirds of women are diagnosed during later stages when 5-year survival rates are only 20% to 30%. However, when ovarian cancer is diagnosed early, the long-term survival rate is almost 90% (1-3).

Lysophosphatidic acid (LPA) is a bioactive lipid that has been shown in preliminary studies to be a promising early diagnostic and prognostic biomarker for EOC (4, 5). LPA exerts its effects upon binding to cognate G protein–coupled receptors encoded by the endothelial cell differentiation gene (Edg) family members. Several high-affinity LPA receptors have been identified, including Edg-2/LPA1, Edg-4/LPA2, and Edg-7/LPA3. Following receptor binding, LPA elicits a diverse range of biological responses, including platelet aggregation, smooth muscle contraction, mitogenesis, and cell shape changes (6).

LPA plays a unique role in the pathobiology of ovarian cancer. Ascitic fluid from the peritoneal cavity of patients with ovarian cancer induces a robust mitogenic response in EOC cells, and LPA has a significant role in this response (6, 7). Interestingly, LPA levels are substantially higher (2-80 μmol/L) in ascites (7, 8) and plasma (3, 4, 9) from patients with early- and late-stage ovarian cancer when compared with levels in healthy subjects or in patients with breast cancer and leukemia. By comparison, activated platelets produce and release lysophospholipids (10), resulting in circulating serum LPA levels of 1 to 5 μmol/L (11). Although LPA is not secreted at significant levels by normal ovarian epithelial cells, EOC cells produce substantially higher levels of LPA. LPA can also stimulate some EOC cells to produce LPA, generating autocrine loops that perpetuate and amplify oncogenic pathways (12, 13). Finally, the LPA receptors, LPA2 and LPA3, were overexpressed in EOC cells when compared with normal cells (12, 14-16), and the activity of autotaxin/lysophospholipase D, an enzyme involved in LPA production, was elevated in ovarian cancer ascites (17).

Another well-recognized role of LPA is the ability to activate RhoA, a small GTPase that mediates changes in actin microfilament organization necessary for adhesion, motility, and cell shape changes. RhoA activates mDia and ROCK1/Rho-kinase to regulate the formation of stress fibers and focal

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adhesions. ROCK has been shown to directly phosphorylate the regulatory light chain of myosin II as well as myosin phosphatase, promoting RhoA-regulated actomyosin contractility. Another important player in mediating actomyosin contractility is myosin light chain kinase (MLCK), a Ca^{2+}-/calmodulin-regulated enzyme that directly phosphorylates regulatory light chains. The balance between MLCK activity (of ROCK and MLCK) and myosin phosphatase activity coordinates regulatory light chain phosphorylation (18).

Matrix metalloproteinase-2 (MMP-2) is present in ovarian cancer ascites, cells, and tissue in which its proteolytic activity is thought to play an important role in i.p. invasion. Ovarian cancer cells respond to LPA by inducing the processing of pro-–metalloproteinase-2 (pro–MMP-2) to its active form, thereby potentiating MMP-dependent cellular migration and invasion (19). MMPs are zinc-dependent endopeptidases that are activated when thezymogen (pro–MMP) is processed at the cell surface (20) via the formation of a ternary complex consisting of the zymogen, tissue inhibitor of metalloproteinase-2 (TIMP-2), and membrane type 1 (MT1)-MMP (21). An adjacent MT1-MMP molecule not bound to TIMP-2 then cleaves the pro–MMP-2 molecule within this complex, yielding an intermediate form. The intermediate MMP-2 species is finally cleaved via a concentration-dependent autolytic event, requiring another MMP-2 molecule, that generates active MMP-2 (22).

Because LPA can regulate both the actin cytoskeleton and pro–MMP-2 activation, we tested the hypothesis that LPA modulates MMP-2 activity via changes in actin microfilament organization. We report that LPA exposure resulted in sustained disruption of stress fibers and increased peripheral filament bundling. Correspondingly, RhoA activity was significantly repressed after LPA treatment. Furthermore, LPA treatment increased MMP-2 levels, promoting pro–MMP-2 activation. Restoration of RhoA activity by expression of the constitutively active mutant, RhoA(G14V), resulted in the down-regulation of LPA-induced pro–MMP-2 activation. Our findings allowed us to construct a model in which LPA plays an active role in EOC dissemination by concurrently promoting loss of adhesion and MMP activation, thereby potentiating metastasis.

Results
LPA Promotes Stress Fiber Disassembly and Enhances Peripheral Filament Formation

LPA mediates changes in actin microfilament organization by activating the small GTPase, RhoA (23, 24). To analyze the effects of LPA on actin cytoskeleton morphology, EOC cells were treated with pathophysiologic levels of LPA (80 μmol/L) for 5 min, and then stained with Texas red–phalloidin to visualize actin filaments. Cells were cultured at high density (confluent) and low density (no cell-cell contacts). When EOC cells were plated at a low density, stress fibers were present in vehicle- and LPA-treated cells (Fig. 1). However, when EOC cells were plated at a high density, LPA exposure began to trigger the loss of stress fibers, as well as an increase in peripheral filaments when compared with vehicle-treated cells (Fig. 1). Some loss of stress fibers was first visible after 5 min of exposure to LPA, but it was not a complete loss (Fig. 1). This disassembly of stress fibers increases with prolonged exposure to LPA, and was very prominent by 24 h (see Fig. 4B). Because LPA-induced changes in the actin cytoskeleton of EOC cells were distinct from other epithelia, in which stress fibers form in response to LPA, phalloidin immunofluorescence was evaluated in three EOC cell lines: DOV13, OVCAR3, and OVCA429. Cells were treated with either vehicle (0.1% BSA/PBS) or 80 μmol/L of LPA for 5 min (arrowheads, peripheral actin filaments; arrows, stress fibers; bar, 100 μm).

FIGURE 1. LPA triggers loss of stress fibers and an increase in peripheral actin filament bundles. Texas red–phalloidin immunofluorescence (n = 3) images depict actin microfilament organization in three EOC cell lines: DOV13, OVCAR3, and OVCA429. Cells were treated with either vehicle (0.1% BSA/PBS) or 80 μmol/L of LPA for 5 min (arrowheads, peripheral actin filaments; arrows, stress fibers; bar, 100 μm).
was then used to solubilize membrane-associated proteins (TX).
The remaining insoluble pellet consisting of proteins associated
with the cytoskeleton (P) was solubilized in buffer containing
SDS. Consistent with the loss of stress fibers seen using
phalloidin immunofluorescence, the amount of vinculin and
talin associated with the cytoskeleton (Fig. 2A, top, lane 12)
was severely diminished after 120 min of exposure to LPA
compared with time 0 (Fig. 2A, lane 3). Densitometric analysis
was done to quantitate the distribution of vinculin and talin in
the various detergent pools in response to LPA treatment over

![Image](https://mcr.aacrjournals.org/content/mcr/5/2/123.full)

**FIGURE 2.** LPA induces focal adhesion disassembly and represses RhoA activity. A. Focal adhesion components shift from the cytoskeletal to the cytoplasmic pool after LPA treatment. Top, anti-talin and anti-vinculin immunoblots using lysates from cells extracted sequentially with buffers containing the detergents saponin (S), Triton X-100 (TX), and SDS (P). Cells were exposed to 80 μmol/L of LPA for the times indicated before sequential detergent extraction was done. Bottom, corresponding densitometric analysis of talin and vinculin expression in the saponin-, Triton X-100–, and SDS-soluble pools. For each time point, talin and vinculin expression was represented as a percentage of total protein expression (sum of density from all detergent pools). Columns, means relative to time 0 min; bars, SE (n = 6). Columns labeled with different letters are statistically significant as analyzed by repeated measures ANOVA, followed by Tukey’s multiple comparison test (P < 0.05). B. Cellular RhoA-GTP bound to RBD-agarose was assayed in cells treated with 80 μmol/L of LPA for 0, 30, 120, and 300 min. RhoA activity was quantified as the amount of RhoA bound to RBD normalized to the amount of RhoA in input cell lysates. Points, mean RhoA activity values relative to the activity at 0 min; bars, SE (n = 3). Student’s t test was done relative to time 0 (a, P < 0.01; b, P < 0.05). C. Representative anti-RhoA immunoblot (n = 3) showing endogenous levels of RhoA-GTP acquired from RBD-agarose affinity-capture assays using lysates from cells treated with 80 μmol/L of LPA for 0, 30, 120, and 300 min (left). The corresponding input lysates were also immunoblotted with anti-RhoA as a loading control (right).
the time course. Talin levels increased 1.4-fold \( (P < 0.05) \) and 1.7-fold \( (P < 0.001) \) in the saponin-soluble pool after 30 and 120 min of LPA treatments, respectively (Fig. 2A, top). Also, talin levels decreased by 36\% \( (P < 0.05) \) and 45\% \( (P < 0.01) \) in the insoluble pool (SDS) after 30 and 120 min of LPA treatments, respectively (Fig. 2A, top). Correspondingly, after 120 min of exposure to LPA, vinculin levels increased 2-fold \( (P < 0.01) \) in the saponin-soluble pool, and decreased 66\% \( (P < 0.001) \) in the insoluble pool (Fig. 2A, bottom). Talin and vinculin levels in the Triton-soluble pool did not change in a statistically significant manner over the time course of LPA treatment (Fig. 2A). These results were consistent with the phalloidin immunofluorescence data and suggest that LPA initiates the stress fiber and focal adhesion disassembly. Furthermore, the loss of stress fibers suggests that the RhoA/ROCK pathway is down-regulated upon LPA stimulation.

**LPA Attenuates RhoA Activity**

Because the loss of stress fibers upon LPA stimulation was indicative of possible repression of the RhoA pathway, experiments were done to evaluate the effects of LPA on RhoA activity. RhoA activation assays were done using the Rhotekin Rho-binding domain (RBD) conjugated to agarose beads. Rhotekin, a Rho effector protein, binds only to Rho-GTP (active Rho) and not Rho-GDP (inactive Rho; ref. 25). RBD-agarose was used to affinity-capture Rho-GTP from cells treated with 80 \( \mu \text{mol/L} \) of LPA for 0, 30, 120, and 300 min. As shown in Fig. 2B, RhoA activity decreased in a statistically significant manner to 51\% after 120 min of exposure to LPA (Fig. 2B and C, compare lanes 1 and 3) and then to 30\% after 300 min (Fig. 2B and C, compare lanes 1 and 4) relative to time 0 \( (P < 0.01 \) and \( P < 0.05 \), respectively).

The levels of inactive RhoA may also be examined by assaying the levels of RhoA/Rho GDP dissociation inhibitor complexes (26). The GDP dissociation inhibitors are thought to inhibit Rho GTPase activity by antagonizing the actions of guanine nucleotide exchange factors and GTPase-activating proteins, and by controlling the translocation of the GTPase from the cytosol to the membrane (27). The Rho GDP dissociation inhibitor was immunoprecipitated from lysates prepared after DOV13 cells were treated with 80 \( \mu \text{mol/L} \) of LPA for 0, 30, 120, and 300 min. However, no dramatic change in the amount of RhoA associated with Rho GDP dissociation inhibitor was observed for up to 300 min after LPA treatment when compared with time 0 (data not shown).

Collectively, the results indicate that LPA exposure significantly represses RhoA activity through a pathway that was not mediated by Rho GDP dissociation inhibitor. The down-regulation of RhoA activity was consistent with the loss of stress fibers and the dissociation of focal adhesion components from the cytoskeleton observed.

**LPA Up-Regulates MMP-2, and RhoA Activity Down-Regulates LPA-Induced Pro–MMP-2 Activation**

Pro–MMP-2 activation may occur by several mechanisms that affect the trimeric complex formation required for zymogen processing at the cell surface. These mechanisms include the up-regulation of MMP-2 or MT1-MMP levels, and the down-regulation of TIMP-2 levels (21). MMP-2 RNA expression from cells treated with LPA was evaluated by reverse transcription-PCR. No change in MMP-2 RNA levels was observed with LPA treatment (Fig. 3A). In addition, MMP-2 expression in whole cell lysates and conditioned medium was assayed by ELISA. LPA stimulation resulted in 1.8-fold higher levels of MMP-2 in cell lysates (Fig. 3B, top) and 1.7-fold higher levels of MMP-2 in conditioned medium (Fig. 3B, bottom) compared with vehicle treatment. In addition, the time course for LPA induction of pro–MMP-2 activation was determined over a 24-h period. Active MMP-2 was detected after 4 h of LPA treatment and continued to increase up to 24 h (Fig. 3C, top). Accumulation of the active MMP-2 species in response to LPA over the time course was quantified by densitometry. Because active MMP-2 was first detected after 4 h of LPA treatment, secretion of active MMP-2 was quantified relative to this time point. Active MMP-2 increased 6.7-fold \( (P < 0.05) \) and 19.8-fold \( (P < 0.001) \) after 12 and 24 h of treatment, respectively (Fig. 3C, bottom). MT1-MMP cell surface expression was also assayed over a time course of LPA treatment for up to 24 h. A representative anti–MT1-MMP immunoblot is shown in Fig. 3D (top). Densitometric analysis revealed that MT1-MMP expression increased 1.8-fold after 6 and 8 h of LPA treatment \( (P < 0.05) \), relative to 0 h (Fig. 3D, middle). The 44 kDa autocatalytic product of MT1-MMP, often detected when enhanced MT1-MMP activity stimulates pro–MMP-2 processing (28-30), was never detected with LPA treatment. Furthermore, secreted TIMP-2 expression did not change dramatically in LPA-treated cells (Fig. 3D). This suggests that LPA stimulates pro–MMP-2 processing by up-regulating MMP-2 and MT1-MMP protein levels, but not by changes in MT1-MMP processing.

Because LPA stimulation significantly decreased RhoA GTPase activity, we wanted to determine if increasing RhoA activity by ectopic expression of a constitutively active RhoA mutant, RhoA(G14V), would repress LPA-stimulated pro–MMP-2 activation. DOV13 cells were transiently transfected with either a RhoA(G14V) construct or the vector, pcDNA3.1(+), and then treated with either vehicle or LPA. Interestingly, cells transfected with RhoA(G14V) exhibited a decrease in LPA-induced pro–MMP-2 activation when compared with cells transfected with empty vector (Fig. 3E, top, compare lanes 2 and 4). Densitometric analysis revealed that RhoA(G14V) transfection reduced LPA-induced MMP-2 activation by 90\% \( (P < 0.05) \) relative to vector-transfected cells (Fig. 3E, bottom).

**ROCK Activity Inhibits LPA-Stimulated Pro–MMP-2 Activation and Stress Fiber Disassembly**

Because expression of RhoA(G14V) negatively regulates pro–MMP-2 processing, the effects of inhibiting ROCK and MLCK (two enzymes that regulate actin microfilament organization) on MMP processing were evaluated.

Regulatory light chain phosphorylation has been shown to be both necessary and sufficient for the formation of stress fibers and focal adhesions in 3T3 fibroblasts. Both MLCK and ROCK are known to directly phosphorylate regulatory light chains (31). Given that loss of central stress fibers and increased peripheral actin filament bundling was observed upon LPA treatment, the effect of inhibiting MLCK and ROCK using ML-7 and...
Y-27632, respectively, on LPA-stimulated pro–MMP-2 activation was determined. Quiescent DOV13 cells were treated with either Y-27632 or ML-7, in the presence or absence of LPA, and conditioned medium was subjected to gelatin zymography to evaluate pro–MMP-2 processing. When cells were treated with increasing concentrations of Y-27632 in conjunction with 80 μmol/L of LPA (Fig. 4A, top left, lanes 4-6), we observed a dose-dependent increase in pro–MMP-2 activation when compared with LPA treatment alone (Fig. 4A, top left, lane 2). Treatment with 10 μmol/L of Y-27632 and LPA increased MMP-2 activation by 1.8-fold (P < 0.05) when compared with LPA treatment alone (Fig. 4A, top, compare columns 1 and 4).

In addition, inhibition of ROCK with Y-27632 decreased central stress fibers and enhanced LPA-induced loss of stress fibers and focal adhesions (Fig. 4B). In contrast, inhibition of MLCK activity did not significantly affect LPA-induced pro–MMP-2 activation (Fig. 4A, bottom). Corresponding densitometric analysis of the active MMP-2 bands from gelatin zymograms over the time course of LPA treatment (bottom) showed that LPA-stimulated MMP-2 activation (white column) did not change in a statistically significant manner with any dose of ML-7 (gray columns) used (Fig. 4A, bottom). Furthermore, MLCK inhibition (with ML-7) decreased peripheral actin filament bundles compared with control cells and attenuated LPA-induced peripheral actin filament bundling when compared with cells treated with LPA alone (Fig. 4B).
Collectively, these results suggest that, in DOV13 cells, MLCK plays a role in peripheral actin filament formation, whereas ROCK plays a role in central stress fiber formation. Also, disruption of ROCK function and not MLCK function enhances LPA-stimulated pro–MMP-2 activation.

Disruption of Actin Filaments Augments LPA-Induced Pro–MMP-2 Activation, but Stabilization of Actin Filaments Represses Pro–MMP-2 Activation

Because LPA induces pro–MMP-2 activation and changes in the actin cytoskeleton, the effects of disrupting versus stabilizing actin filaments on pro–MMP-2 activation were evaluated.

Quiescent DOV13 cells were treated with cytochalasin D (CyD), which disrupts actin filaments, for 24 h and then conditioned medium was subjected to gelatin zymography. Treatment of cells with CyD alone was sufficient to stimulate processing of pro–MMP-2 to its intermediate form (Fig. 5A, top left, lane 3). CyD also enhanced LPA-regulated pro–MMP-2 activation in a concentration-dependent manner (Fig. 5A, top left, lanes 4-6) when compared with LPA treatment alone (Fig. 5A, top left, lane 2). When compared with LPA treatment alone, the addition of 1 and 3 μmol/L of CyD increased LPA-induced active MMP-2 secretion by 1.8-fold and 2.0-fold (P < 0.01), respectively (Fig. 5A, top right, compare column 1 with columns 3 and 4).

**FIGURE 4.** ROCK and MLCK play different roles in pro–MMP-2 processing and actin microfilament organization. **A.** Gelatin zymography of conditioned medium from cells treated with Y-27632 (0.2, 1, and 10 μmol/L) and ML-7 (0.2, 5, and 10 μmol/L) in the presence or absence of 80 μmol/L LPA for 24 h (left). Graphs depicting corresponding densitometric analysis of the active MMP-2 bands from LPA-treated cells (1) and cells treated with increasing concentrations of inhibitor (Y-27632 or ML-7) and LPA (2-4) on gelatin zymograms (right). Columns, means relative to LPA treatment alone; bars, SE (n = 3 for each inhibitor). Columns labeled with different letters are statistically significant as analyzed by repeated measures ANOVA, followed by Tukey’s multiple comparison test (P < 0.05). **B.** Texas red–phalloidin immunofluorescence of cells treated with the following for 24 h: vehicle (0.1% BSA/PBS/DMSO); 80 μmol/L of LPA; 10 μmol/L of ML7; 10 μmol/L of ML-7/80 μmol/L of LPA; 10 μmol/L of Y-27632; and 10 μmol/L of Y-27632/80 μmol/L of LPA (n = 3). Arrows, central stress fibers; arrowheads, peripheral actin filaments; bar, 100 μm. Bottom, bright-field images of cells treated with either vehicle (0.1% BSA/PBS) or 80 μmol/L LPA for 24 h (n = 3). Note that LPA-treated cells round up and control cells are more flattened (arrowheads, single cell within each bright-field image; bar, 50 μm).
Furthermore, treatment with cyD disrupted central stress fibers and peripheral filaments, with some cortical filaments still visible at the cell periphery (Fig. 5B). Peripheral filament staining was more intense in cells treated with both cyD and LPA than in cells treated with cyD alone (Fig. 5B). CyD was much more efficient at disrupting central stress fibers than Y-27632, which may explain why cyD treatment alone was sufficient to promote more robust processing of pro–MMP-2 to its intermediate form.

The effects of restoring stress fiber formation on LPA-triggered pro–MMP-2 activation were also assessed by treating cells with phalloidin. Phalloidin treatment diminished LPA-stimulated pro–MMP-2 activation in a dose-dependent manner (Fig. 5A, middle left, lanes 4-6) when compared with LPA treatment alone (Fig. 5A, middle left, lane 2). Treatment with 20 μg/mL of phalloidin decreased LPA-dependent pro–MMP-2 activation by 40% (P < 0.05) when compared with LPA treatment alone (Fig. 5A, middle right, compare columns).

**FIGURE 5.** Effects of cytoskeletal modulators on pro–MMP-2 processing and actin microfilament organization. A. Gelatin zymography of conditioned medium from cells treated with cyD (cyD, 0.1, 1, and 3 μmol/L), phalloidin (Phall, 2, 5, and 20 μmol/L), and nocodazole (Noc, 10 μmol/L) in the presence or absence of 80 μmol/L LPA for 24 h (left). Conditioned medium from cells treated with vehicle (BSA/PBS/DMSO) was used as a control. Right, graphs depicting corresponding densitometric analysis of the active MMP-2 bands from cells treated with LPA alone (1) and cells treated with both inhibitor (cyD, Phall, or Noc) and LPA (2-4). Columns, means relative to LPA treatment alone; bars, SE (n = 3). For cyD and phalloidin treatment, columns labeled with different letters are statistically significant as analyzed by repeated measures ANOVA, followed by Tukey’s multiple comparison test (P < 0.05). For nocodazole treatment, paired Student’s t test was done (*, P < 0.05). B. Texas red–phalloidin immunofluorescence showing actin microfilament organization of quiescent cells treated with LPA alone (1) and cells treated with both inhibitor (cyD, LPA, or Phall) and LPA (2-5). Arrows, central stress fibers; arrowheads, peripheral actin filaments; and arrowheads in Noc panels, focal contacts (bar, 100 μm).
Phalloidin treatment also restored stress fiber formation in LPA-treated cells, although the stress fibers seemed thinner when compared with those in vehicle-treated cells (Fig. 5B).

To ensure that the induction of pro–MMP-2 processing was not simply due to the disruption of the cytoarchitecture of the cell, we also treated cells with nocodazole, a compound that destabilizes microtubules. Unlike cyD, nocodazole treatment alone had no effect on pro–MMP-2 processing (Fig. 5A, bottom left, lane 3), but significantly inhibited LPA-stimulated pro–MMP-2 activation (Fig. 5A, bottom left, lane 4) when compared with LPA treatment alone (Fig. 5A, bottom left, lane 2). Nocodazole treatment repressed LPA-induced active MMP-2 secretion by 80% (P < 0.05) relative to LPA treatment alone (Fig. 5A, bottom, compare columns 1 and 2). Interestingly, nocodazole treatment induced the formation of peripheral focal contacts and restored some stress fiber formation in LPA-treated cells (Fig. 5B). Collectively, these data suggest that loss of stress fibers and focal adhesions enhances LPA-induced pro–MMP-2 processing, whereas stabilization of actin filaments represses LPA-induced pro–MMP-2 processing.

**ROCK Activity Is Required for LPA-Induced Haptotactic Migration in Cells Cultured at Low Density**

Our results show that when cells were cultured at a high density, LPA down-regulates RhoA/ROCK activity, which contributes to pro–MMP-2 activation. However, when cells were cultured at a low density, stress fibers were present even after LPA stimulation (Fig. 1). We previously reported that LPA induces EOC haptotactic cell migration (19). Because stress fibers are present in cells treated with LPA at a low density, we next wanted to determine if the RhoA/ROCK pathway plays a role in LPA-induced EOC cell migration. DOV13 cells were cultured at a low density on glass coverslips coated with colloidal gold and type I collagen, and the area of phagokinetic tracks of individual migrating cells was quantitated. LPA stimulated EOC migration 1.9-fold (P < 0.001) relative to vehicle treatment, and inhibition of ROCK activity with Y-27632 (1, 10, and 20 μmol/L) significantly repressed LPA-induced cell migration (in a dose-dependent manner) down to basal levels (Fig. 6B). The stimulation of cell migration by LPA, in addition to the repression of LPA-stimulated cell migration by Y-27632, could be visualized by the images of the phagokinetic tracks (black area on the colloidal gold background) made by the cells from each treatment (Fig. 6A).

Taken together, these results show that LPA exerts differential effects on actin microfilament organization depending on EOC cell density. These differential effects may promote metastatic dissemination by promoting the loss of focal adhesion and pro–MMP-2 activation in cells at a high density, and migration in cells at a low density.

**Discussion**

LPA is thought to play a unique role in the pathobiology of ovarian cancer because levels of this bioactive lipid are elevated (2-80 μmol/L) in the plasma and ascites of patients with early- and late-stage ovarian cancer (4, 7, 8). In ovarian cancer cells, LPA can induce LPA production, generating autocrine loops that promote tumorogenic pathways such as survival, proliferation, migration, invasion, and angiogenesis (6). The studies presented here investigated the relationship between LPA-mediated changes in actin microfilament organization and MMP activation. We found that, in EOC cells cultured to a high density, LPA induced the loss of stress fibers and focal adhesions, as well as the formation of peripheral actin filaments. Consistent with the observed disassembly of stress fibers, RhoA activity decreased significantly in response to LPA. These findings show that LPA stimulation attenuated RhoA activity in DOV13 cells, in contrast with results reported for other quiescent normal and malignant epithelium, which form stress fibers as a result of RhoA activation by LPA (32-34).

LPA has different effects on the actin cytoskeleton of DOV13 cells cultured at a low density (stress fibers remain) versus high density (loss of stress fibers). The unique changes in actin microfilament organization induced by LPA may be due to the fact that EOC cells possess both epithelial and...
mesenchymal characteristics (35). Ovarian surface epithelia express smooth muscle actin, a mesenchymal marker, and can contract a collagen gel lattice in vitro (36). EOC cells also produce LPA and overexpress LPA receptors, which may affect microfilament organization (6, 7). It will be critical to elucidate the factors, such as cadherins, that dictate how LPA differentially modulates RhoA activity in high-density versus low-density conditions. Cadherin family members have been implicated in the regulation of MMP expression and invasive potential of tumor cells (37-39).

High-density cultures can be thought of as a simplified two-dimensional model of cells within a solid tumor, whereas low-density cultures can be thought of as metastatic populations that may exist as single cells or as small colonies. We propose a model in which LPA is elevated in the ovarian cancer cell microenvironment and has different effects on EOC cells depending on the pathobiological status of malignant epithelia. LPA may promote loss of stress fibers and focal adhesions as well as pro–MMP-2 activation in cells within a solid tumor. Loss of adhesion would facilitate the dissociation of single cells or small groups of cells from the primary tumor, allowing EOC cells to attach to the i.p. cavity and, hence, metastasize. In fact, we have reported that LPA stimulated MMP-dependent EOC haptotactic migration and invasion (19). In the current study, stress fibers and focal adhesions were present after LPA treatment of EOC cells cultured at a low density, indicating that RhoA was active. Furthermore, Rho-kinase/ROCK function is required for LPA-induced haptotactic migration on thin layer type I collagen.

Several studies have shown that disruption of the actin cytoskeleton by cyD promotes the activation of pro–MMP-2 (40-42). LPA may work through a similar mechanism to activate pro–MMP-2 by triggering the disassembly of stress fibers. In DOV13 cells, ROCK regulates central stress fiber formation, whereas MLCK regulates peripheral filament formation. Inhibition of MLCK activity did not significantly affect LPA-mediated pro–MMP-2 activation, but if ROCK activity is inhibited, further augmenting LPA-induced loss of stress fibers, then an increase in LPA-triggered pro–MMP-2 activation is observed. Also, counteracting LPA repression of RhoA by expression of the constitutively active mutant, RhoA(G14V), down-regulated pro–MMP-2 activation.

Mechanical stress relayed by the extracellular matrix and the organization of the actin microfilament system has been shown to play a role in MMP expression and activation. When fibroblasts were grown on substrates that promote cell spreading, such as attached collagen substrates, stress fibers and focal adhesions form and most of the MMP produced is inhibited, further augmenting LPA-induced loss of stress fibers, then an increase in LPA-triggered pro–MMP-2 activation is observed. Also, counteracting LPA repression of RhoA by expression of the constitutively active mutant, RhoA(G14V), down-regulated pro–MMP-2 activation.

When LPA treatment relaxes mechanical tension in EOC cells by inducing the disassembly of stress fibers and focal adhesions. In addition, LPA treatment up-regulated MMP-2 expression, which can facilitate trimolecular complex formation and autolytic cleavage of the intermediate MMP-2 form required for zymogen activation at the cell surface.

Collectively, our data are consistent with a model in which LPA promotes EOC metastasis by triggering loss of adhesion and activation of MMPs, or haptotactic migration. Further elucidation of mechanisms by which LPA induces modifications of the microfilament system and processing of proteinases may lead to new insights in ovarian oncogenesis and the design of novel therapeutic targets.

Materials and Methods

Cell Culture, Treatments, and Transfections

The DOV13 cell line, a generous gift from Dr. Robert Bast, Jr. (M.D. Anderson Cancer Center, Houston, TX), was cultured as previously described (51). OVCAR3 and OVCA429 cells were grown in RPMI 40 supplemented with 20% fetal bovine serum and MEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), respectively. For all experiments, cells were cultured to confluency and serum-starved for 24 h before treatment, except when low-density conditions were specified, cells were plated at 1.5 × 105 cells/mL. Cells were pretreated with phallolidin (Molecular Probes, Eugene, OR) during the 24-h serum starvation period. For all other inhibitors, cells were pretreated with inhibitor for 1 h before culturing cells in the presence of inhibitor and 80 μmol/L of 18:1 LPA (Avanti Polar Lipids, Alabaster, AL) for 24 h. ML-7, nocardazole, and cyD were from Sigma-Aldrich (St. Louis, MO), whereas Y-27632 was from EMD Biosciences (San Diego, CA). For transient transfections, cells were maintained in Opti-MEM after reaching confluency, and then transfected using Lipofectamine 2000 Reagent (Invitrogen) and 1.6 μg of DNA [pcDNA3.1(+) or pcDNA3.1(+)_RhoA(G14V)] for each well of a 12-well plate, according to the instructions of the manufacturer. A second transfection was done 24 h later, and cells were treated on the same day with 80 μmol/L of LPA for 24 h. Transfection efficiencies were determined by cotransfection of the pEGFP construct (0.1 μg).

Immunofluorescence and Imaging Techniques

Cells were cultured on glass coverslips and fixed in 4% paraformaldehyde in 20 mmol/L of HEPES (pH 7.4) and 150 mmol/L of NaCl for 20 min, permeabilized in 0.1% Triton X-100 in PBS for 10 min, blocked with 1% bovine serum albumin (BSA)/PBS for 1 h, and then incubated at room temperature for 1 h with Texas red–phalloidin (Molecular Probes) at 1:100 in blocking solution. Images were acquired on a Nikon Eclipse TE2000-U microscope using the SPOT digital camera and Metamorph 5.0 software.

Gelatin Zymography

To analyze pro–MMP-2 processing, cells were cultured to confluency in 12-well plates (200,000 cells/mL), grown overnight, and then treated as described above. Conditioned medium was subjected to SDS-PAGE (52) under nonreducing conditions,
using 9% polyacrylamide gels containing 0.1% gelatin. Gels were washed twice in 2.5% Triton X-100 for 30 min, incubated in 20 mmol/L of glycine, 10 mmol/L of CaCl2, and 1 μmol/L of ZnCl2 (pH 8.3) at 37°C for 24 h, and then stained in Coomassie blue to detect areas of gelatinolytic activity.

**RhoA Activation Assay**

To assess RhoA activation, the amount of RhoA-GTP bound to the Rhotekin RBD was determined using the Rho Activation Assay Kit (Upstate Biotechnology, Lake Placid, NY) according to the instructions of the manufacturer. Briefly, DOV13 cells were cultured to confluency, made quiescent, and then treated with the instructions of the manufacturer. Briefly, DOV13 cells were the Rhotekin RBD was determined using the Rho Activation RhoA Activation Assay Coomassie blue to detect areas of gelatinolytic activity.

**Densitometry**

Densitometric analysis was done on (a) the active MMP-2 bands from gelatin zymograms; (b) anti-MT1-MMP, anti-talin, and anti-vinculin immunoblots, (c) and RhoA activity results from at least three independent experiments using Molecular Analyst 1.5 and Image J 1.34s software. For gelatin zymograms, the densities of the active MMP-2 bands from different treatment conditions were compared, and these analyses assumed that all bands were within the linear range. All statistical analyses were done using Prism 4 software.

**MMP-2 ELISA**

Analysis of pro- and active MMP-2 protein concentration in whole cell lysates and conditioned media were done using the Quantikine MMP-2 (total) ELISA kit (R&D Systems, Minneapolis, MN) according to the instructions of the manufacturer. Transfection-conditioned media were concentrated 3-fold. Samples from three independent experiments were assayed in duplicate and absorbance values were acquired on a Spectramax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA).

**Reverse Transcription-PCR**

Total RNA was isolated from DOV13 cells using Qiagen’s RNeasy kit according to the instructions of the manufacturer (Qiagen, Valencia, CA). Reverse transcription reactions were carried out using 2 μg of RNA (DNasel-treated) and the RETROscript kit according to the protocols of the manufacturer. The primers used to amplify MMP-2 were: 5'-TTGAG AAGGATGGCAGAATCGGCT-3' and 5'-AGAGCCTCTC- GAATGGCCTTTGAT-3'. The primers used to amplify RPL19 were: 5'-ATGAAATCGCCAATGCCAATCCC 3' and 5'-CTGCTTTCAGCTTGAGATGTGTT-3'.

**Cellular Extracts, Cell Surface Biotinylation, and Immunoblotting**

For detergent extractions, cells were extracted with saponin buffer [0.1% saponin, 10 mmol/L Tris-HCl (pH 7.5), 140 mmol/L NaCl, 2 mmol/L EGTA, 5 mmol/L EDTA, 1 mmol/L Na2VO4, 25 mmol/L NaF] for 30 min, scraped, and centrifuged at 15,000 rpm for 30 min. The supernatant was designated the saponin-soluble pool (S) and the pellet was resuspended in Triton X-100 buffer (same composition as saponin buffer, but with 1% Triton X-100 instead of saponin). The Triton X-100 lysate was centrifuged for 30 min, and the remaining supernatant was designated the Triton-soluble pool (T). The remaining pellet (P) was resuspended in Laemmli sample buffer. Whole cell lysates were prepared by incubating cells in Laemmli sample buffer for 20 min at 4°C and then boiling for 5 min. Biotinylation of cell surface proteins was done by incubating cells with 0.5 mg/mL of Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) for 30 min at 4°C. Free biotin was then quenched with 100 mmol/L of glycine and cells were lysed [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, and 0.1% SDS] and then clarified by centrifugation. Biotinylated proteins were isolated by incubation with streptavidin beads (Pierce) at 4°C for 16 h and then boiled in Laemmli sample buffer.

Cell extracts were subjected to SDS-PAGE on a 7% or 15% polyacrylamide gel. Proteins were transferred to nitrocellulose, blocked for 1 h in either TBBS (0.005% Tween 20 in PBS) or 2% milk/TBBS, and blotted with anti-vinculin (Sigma), anti-talin (Sigma), or anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,000 dilutions. Blots were washed thrice for 5 min in TBBS, incubated with anti-mouse antibody conjugated to horse-radish peroxidase (1:5,000) for 1 h, washed twice for 5 min in TBBS and twice for 5 min in PBS, and then developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**Haptotactic Migration Assay**

For cell migration assays, cells were plated at 1.5 x 104 cells/mL (low density conditions) onto 25 mm square glass coverslips coated with type I collagen (10 μg/mL) and gold chloride trhydrate (Sigma) as described by Albrecht-Buehler (53). Cells were allowed to adhere for 2 h before treatment and then allowed to migrate for 24 h before fixation with 0.1% formaldehyde. The area of a phagokinetic track from a single cell was quantified using a Wescott Verta VU 7000 series inverted microscope and NIH Scion Image software (version 4.0). The areas of 50 phagokinetic tracks per coverslip, from duplicate sets of coverslips for each treatment, were averaged for each experiment; three independent experiments were done.

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**References**


Lysophosphatidic Acid Down-Regulates Stress Fibers and Up-Regulates Pro–Matrix Metalloproteinase-2 Activation in Ovarian Cancer Cells

Thuy-Vy Do, Jay C. Symowicz, David M. Berman, et al.


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