Lysophosphatidic Acid Is a Major Regulator of Growth-Regulated Oncogene α in Ovarian Cancer

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Abstract

Growth-regulated oncogene α (GROα), a member of the chemokine superfamily, is commonly expressed in transformed cells and contributes to angiogenesis and tumorigenesis. Here, we report that increased GROα levels are detected in the plasma and ascites of ovarian cancer patients. Ovarian cancer cell lines in culture express and secrete GROα. However, when they are starved in serum-free medium, ovarian cancer cells ceased producing GROα, suggesting that GROα is not constitutively expressed but rather is produced in response to exogenous growth factors in ovarian cancer cells. The prototype peptide growth factors present in serum such as platelet-derived growth factor, insulin-like growth factor I, and insulin do not stimulate GROα production by ovarian cancer cells. In contrast, lysophosphatidic acid (LPA), a glycerol backbone phospholipid mediator present in serum and ascites of ovarian cancer patients, is a potent inducer of GROα expression in ovarian cancer cell lines. Treatment of ovarian cancer cells with LPA leads to transcriptional activation of the *GROα* gene promoter and robust accumulation of GROα protein in culture supernatants. The action of LPA on GROα expression is mediated by LPA receptors, particularly the LPA_2 receptor in that ectopic expression of these receptors restores the LPA-dependent GROα production in nonresponsive cells. Down-regulation of LPA_2 expression by small interfering RNA (siRNA) in ovarian cancer cells desensitizes GROα production in response to LPA. The effect of serum on GROα production is also significantly decreased by siRNA inhibition of LPA_2 expression. These studies identify LPA as a primary regulator of GROα expression in ovarian cancer. (Cancer Res 2006; 66(5): 2740-8)

Introduction

The ELR-CXC chemokines with the amino acid sequence glutamic acid-leucine-arginine (the ELR motif) at the NH_2-terminal domain of the ligands, including CXCL1 or growth-regulated oncogene α (GROα), are neutrophil-activating chemokines and serve as potent angiogenic factors (1, 2). GROα was originally identified by its constitutive overexpression in transformed Chinese hamster fibroblasts (3). Exogenously applied GROα exhibits growth-promoting activity toward melanoma cells and therefore GROα is also called melanoma growth-stimulatory activity (4, 5). Ectopic expression of GROα converts immortalized melanocytes, melanoma precursor cells, to a tumorigenic phenotype, suggesting that GROα may possess transforming activity in certain types of cells (6, 7).

GROα may also contribute to tumorigenesis through its proangiogenic effect (7). The chemokine exerts its biological actions through the G protein–coupled receptor CXCR2 present in granulocytes, endothelial cells, and other cell lineages (8). Interestingly, the Kaposi sarcoma–associated herpesvirus G protein–coupled receptor (vGPCR) is a viral homologue of the human CXCR2 (9). Recent studies of oncogenic mechanisms for the Kaposi sarcoma virus indicate that the vGPCR is sufficient to initiate Kaposi sarcoma–like tumors when targeted to the vascular endothelium of mice (10), highlighting the importance of GROα/CXCR2 signaling in tumor angiogenesis and neoplasia.

Increased GROα expression is frequently detected in melanoma (4, 11), squamous cell carcinoma (12, 13), colon cancer (14), diffuse type of gastric carcinoma (15), and oral cancer (16). However, the mechanism for the deregulated expression GROα in malignant cells is poorly understood. In the current study, we assess the expression and regulation of GROα in ovarian cancer wherein GRO has not been previously studied. Our results showed that many ovarian cancer patients have elevated plasma levels of GROα, which are accompanied by even higher concentrations of GROα in ascites. These results suggest that GROα is generated in ascites, likely by ovarian cancer cells, and migrates to the peripheral circulation. In culture, ovarian cancer cells elaborated GROα. Under serum-free conditions, however, these cells stopped producing GROα, suggesting that the chemokine is not constitutively expressed by ovarian cancer cells but is rather responsive to growth factors in serum. We showed that lysophosphatidic acid (LPA), which is present in serum and ascites of ovarian cancer patients (17), strongly stimulated GROα production in ovarian cancer cells through LPA receptors of the endothelial differentiation gene family (17), particularly the LPA_2 receptor. Down-regulation of LPA_2 expression by small interfering RNA (siRNA) in ovarian cancer cells reduced the responsiveness to LPA and to serum. These results identify the serum lipid constituent LPA as a major regulator of GROα expression in ovarian cancer cells. In light of the widespread distribution of LPA and its receptors, LPA induction of GROα likely represents a general mechanism for GROα deregulation in neoplasia.

Materials and Methods

Reagents. LPA (18:1 and 14:0), phosphatidic acid (dipalmitoyl), and sphingosine 1-phosphate (S1P) were purchased from Avanti Polar Lipids (Alabaster, AL). Before use, these phospholipids were dissolved in PBS containing 0.5% fatty acid–free bovine serum albumin (Roche, Indianapolis, IN). Platelet-derived growth factor (PDGF), epidermal growth factor (EGF),

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fetal bovine serum (FBS), actinomycin D, Ki16425, and anti-flag M2 and anti-β-actin monoclonal antibodies were obtained from Sigma (St. Louis, MO). Pertussis toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA). Insulin-like growth factor I (IGF-I) was from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-Erk antibody and luciferase assay kits were purchased from Promega (San Luis Obispo, CA). Anti-phospho-Akt (Ser^{473}) antibody was from Cell Signaling (Beverly, MA). Anti-CXCR2 neutralizing antibody was obtained from R&D Systems (Minneapolis, MN).

Clinical samples. Plasma and ascitic fluids of ovarian cancer patients were kindly provided for this study by the Basic Biology of Ovarian Cancer PPG Tissue Bank (University of California San Francisco, San Francisco, CA). The patients were diagnosed with ovarian carcinomas at stage III or IV. Ten of the 13 patients were classified as the most common serous type of epithelial ovarian carcinomas, whereas the other three had mixed or undifferentiated ovarian carcinomas. The plasma specimens of normal controls were also provided by the Basic Biology of Ovarian Cancer PPG Tissue Bank. These were healthy female volunteers attending outpatient clinics for routine physical examination.

Cells. The source and maintenance of ovarian cancer cell lines, including OVCAR-3, SKOV-3, Dov-13, and Caov-3, have been described previously (18). The human breast carcinoma cell line MCF-7 was obtained from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-468 and SKBr-3 were provided by Dr. Y. Yu (M.D. Anderson Cancer Center). Breast cancer cell lines were cultured in RPMI plus 10% FBS. All cell lines were frozen at early passages and used for <10 weeks in continuous culture. Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex BioScience, Inc. (Walkersville, MD), and cultured with the EGM-2 complete medium as recommended by the supplier.

Measurement of GROα production by ELISA. Culture supernatants of cell lines treated without or with LPA or other stimuli were collected and analyzed for measuring GROα concentrations by ELISA using the human GROα Quantikine ELISA kit (R&D Systems). Concentrations and quantities of GROα in culture supernatants and clinical samples were calculated by comparing the absorbance of samples to standard curves.

Western blot. Cells were lysed in SDS sample buffer or in ice-cold X-100 lysis buffer [1% Triton X-100, 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 100 mmol/L NaF, 10 mmol/L Na PPi, and protease inhibitor cocktail (Roche)]. Total cellular protein was resolved by SDS-PAGE, transferred to immobilon [poly(vinylidene difluoride)], and immunoblotted with antibodies following the protocols provided by the manufacturers. Immunocomplexes were visualized with an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ) using horseradish peroxidase–conjugated secondary antibodies (Cell Signaling).

Northern blot analysis. Total cellular RNA was extracted from cell lines using the TRIzol reagent, following the instruction of the supplier (Invitrogen, Carlsbad, CA). RNA samples were size-fractionated by formaldehyde/agarose gel electrophoresis, stained with ethidium bromide, and transferred to N^+ hybrid nylon. RNA was immobilized by UV cross-linking, and then prehybridized and hybridized to ^{32}P-labeled cDNA probes as described previously (19). The human LPA_1, LPA_2, and LPA_3 cDNAs were isolated from their expression vectors pcDNA3-Flag-LPA_1, pcDNA3-Flag-LPA_2, and pcDNA3-Flag-LPA_3 (18). The cDNA clone for the human *GROα* gene was obtained from ATCC. Quality of RNA samples was confirmed by rehybridization to the human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA or to the DNA of 18S rRNA (ATCC).

Luciferase assay. The human GROα promoter sequences (−304 to +9, −150 to +9, −84 to +9, and −56 to +9; ref. 11, 20) were cloned from the genomic DNA of OVCAR-3 cells by PCR amplification, verified by automatic sequencing, and inserted into the pGL2-Basic vector (Promega). For luciferase assays, ovarian cancer cell lines were transfected with pGL2-GROα-Luc using Fugene 6 according to the instructions of the manufacturer (Roche). About 48 to 60 hours after transfection, the cells were starved for 24 to 48 hours before stimulation with LPA or vehicle for 6 hours. Cell extracts were prepared and assayed for luciferase activity using the luciferase assay kit from Promega. The luciferase activity was normalized on the basis of the activity of cotransfected β-galactosidase reporter driven by the cytomegalovirus promoter (pCMVβ-gal).

Restoration of LPA-induced GROα production in SKBr-3. To determine whether enforced expression of LPA receptors can restore cytokine production in response to LPA, the nonresponsive cell line SKBr-3 was transfected with each LPA receptor expression vector with LipofectAMINE 2000 according to the protocol of the supplier (Invitrogen). Approximately 60 hours after transfection, the cells were starved in serum-free medium for 36 hours before stimulation with LPA for 24 hours. The conditioned medium was harvested for ELISA quantification of GROα.

siRNA. The human LPA_2 SMARTpool siRNA and the control non-targeting siRNA were obtained from Dharmacon (Lafayette, CO). OVCAR-3 cells were plated in 24-well plates at 1.25×10^5 cells per well in complete medium without antibiotics the day before transfection. The cells were transfected overnight with control or LPA_2-specific siRNA (50 pmol) with LipofectAMINE 2000 (2.5 μL) resuspended in 500 μL of Opti-MEM (Invitrogen). After transfection, the cells were incubated with fresh complete medium for 36 hours for recovery and starved in serum-free medium for 10 hours before stimulation with FBS, LPA, or vehicle. The culture supernatants were collected for ELISA analysis of GROα.

Chemotaxis assay. The migration of HUVEC was measured with modified Boyden chambers as described previously (21). In brief, polycarbonate filters (Neuro Probe, Inc., Gaithersburg, MD) were coated with 10 μg/mL fibronectin (Invitrogen). Conditioned culture medium of ovarian cancer cell lines treated with or without LPA was added to the bottom chambers. Cells (4×10^4 in 100 μL) were loaded into the upper chambers and incubated at 37°C for 4 hours to allow migration. The cells migrated to the bottom side of the filter were fixed, stained, and counted under microscope (21).

Results

The presence of GROα in plasma and ascites of ovarian cancer patients. The expression of GROα has been investigated in melanoma (4, 5, 11), squamous cell carcinoma (12, 13), colon cancer (14), prostate cancer (22), and gastric cancer (15). To explore the role of GROα in ovarian cancer, we set out to determine GROα in ovarian cancer patient samples by ELISA analysis. As shown in Fig. 1*A*, normal controls displayed consistently low levels of GROα (<160 pg/mL) in their plasma samples. However, the concentrations of GROα in ovarian cancer patients varied remarkably. Approximately 40% of patients with ovarian cancer showed dramatically increased GROα concentrations in the plasma with the remaining patients having GROα levels similar to those of normal individuals. To track the origin of the increased GROα in ovarian cancer patients, we compared GROα levels in the plasma and matched ascites samples from ovarian cancer patients. As shown in Fig. 1*B*, GROα levels in ascites were consistently higher than the matched plasma concentrations. For example, the plasma GROα concentration of patient 2 was slightly over 1,000 pg/mL whereas the GROα level in her ascites reached 3,000 pg/mL. These observations are compatible with GROα being produced in ascites, likely by ovarian cancer cells, and migrating from the peritoneal cavity to the circulation as occurs with other tumor markers, such as CA125 (23).

Expression and regulation of GROα in ovarian cancer cells in culture. To study the ability of ovarian cancer cells to elaborate GROα, we measured the levels of GROα in conditioned medium of various ovarian cancer cell lines, including OVCAR-3, Caov-3, SKOV-3 and Dov-13. These cells were maintained with complete medium containing 5% FBS. As shown in Fig. 2*A*, ELISA analysis detected high levels of GROα in the conditioned medium from each of the ovarian cancer cell lines examined. It is apparent that GROα present in the supernatants was secreted from cultured

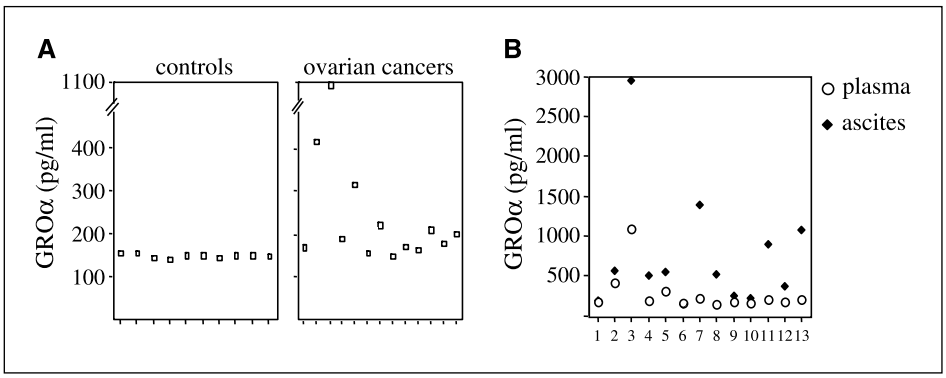

Figure 1. GROα in the plasma and ascites of ovarian cancer patients. The GROα levels (pg/mL) in the plasma of normal female volunteers and in the plasma and ascites of ovarian cancer patients were quantified by ELISA as described in Materials and Methods. *A,* the plasma GROα levels of normal controls (n = 10) were compared with those of ovarian cancer patients (n = 13). *B,* differences between the plasma and the matched ascitic concentrations of GROα in ovarian cancer patients (n = 13).

ovarian cancer cells rather than a contaminant of FBS as the culture medium supplemented with 5% FBS contained extremely low background levels of GROα protein.

To determine mechanisms regulating GROα expression and secretion by ovarian cancer cells, we quantified GROα production in different culture conditions. OVCAR-3 and Caov-3 cells were cultured in complete medium containing 5% FBS for 20 hours (time 0) and then switched to serum-free medium for 40 hours with conditioned medium collected at 20-hour intervals for ELISA analysis (Fig. 2*B*). Interestingly, GROα concentrations in conditioned medium decreased immediately and rapidly when the cells were starved in serum-free medium. By 20 hours in serum-free conditions, GROα levels in the medium were reduced to <20% of the original values at time 0. Ovarian cancer cell lines are generally resistant to serum deprivation–induced cell death as we described previously (17). Both OVCAR-3 and Caov-3 lines remained healthy after 40 hours of incubation in serum-free medium. These cell lines continued to grow albeit slowly in serum-free conditions as determined by quantification of cell numbers (data not shown). Hence, the observed decrease in GROα generation was apparently not due to loss of cells. These results indicate that GROα is not constitutively expressed. Instead, its expression or release by ovarian cancer cells is induced by serum.

Induction of GROα expression by LPA but not serum peptide growth factors. To identify the factor(s) in serum that was responsible for inducing GROα expression in ovarian cancer cells, we assessed the effects of a number of serum-borne peptide growth factors, PDGF, IGF-I, and insulin. None of these peptide growth factors induced significant GROα production in OVCAR-3 or Caov-3 cells as determined by ELISA analysis of culture supernatants (Fig. 3*A*). We next examined the presence of the corresponding receptors for these peptide growth factors. Most ovarian cancer cell lines, including OVCAR-3, Caov-3, and SKOV-3, do not seem to express functional levels of PDGF receptors. As reflected in Fig. 3*B*, treatment of OVCAR-3 or Caov-3 with PDGF did not provoke phosphorylation of Erk or Akt, indicating the lack of functional PDGF receptors in these cells. OVCAR-3 cells also lacked signaling responses to IGF-I whereas Caov-3 cells showed prominent phosphorylation of Akt upon stimulation with IGF-I (Fig. 3*B*). The insulin receptor is present and functional in both OVCAR-3 and Caov-3 cells as insulin stimulated rapid phosphorylation of Erk and Akt. However, only minimally stimulant effect of insulin on GROα generation was observed in OVCAR-3 or Caov-3 cells, suggesting that insulin is not a potent inducer of GROα expression in these cells.

We also examined whether EGF could induce GROα as ovarian cancer cells generally express EGF receptors. EGF stimulated Erk and/or Akt phosphorylation in OVCAR-3, Caov-3 (Fig. 3*B*), and SKOV-3 cells (data not shown), confirming the expression of functional EGF receptors in these cells. EGF induced modest increase in GROα production in OVCAR-3 (from 88.2 to 234.2 pg/mL; Fig. 3*A*) or in SKOV-3 (data not shown), but strongly induced GROα production in Caov-3 (from 219.2 to 1,491 pg/mL; Fig. 3*A*). The variability of the effect of EGF on GROα production may reflect variable expression levels of EGF receptor or differential EGF signaling effects across the different cell lines. Nevertheless, the results indicate that EGF is not a general inducer of GROα production in ovarian cancer cells.

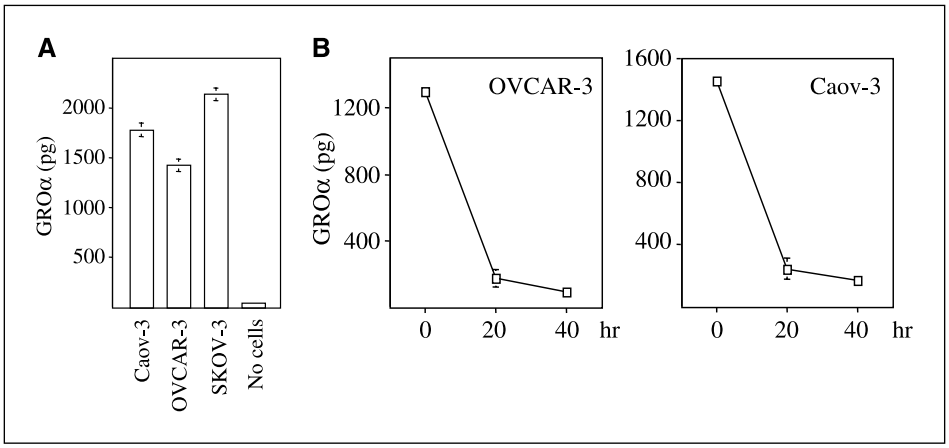

Figure 2. Serum-dependent production of GROα by ovarian cancer cells. *A,* the ovarian cancer cell lines Caov-3, OVCAR-3, and SKOV-3 were cultured in six-well plates (5×10^5 per well) for 1 day in complete medium (RPMI 1640 + 5% FBS) before the culture supernatants were collected for determination of GROα concentrations using ELISA. The FBS-containing medium incubated for the same period of time in the absence of cells was included as a negative control (no cells) to confirm the secretion of GROα from the cultured cells instead of FBS. *B,* OVCAR-3 and Caov-3 cells were first cultured in complete medium for 20 hours (time 0) and then switched to serum-free medium for 40 hours with the conditioned medium collected at 20-hour intervals (time 20 and 40 hours) for ELISA. *Points,* mean of triplicate assays; *bars,* SD. Representative of three independent experiments.

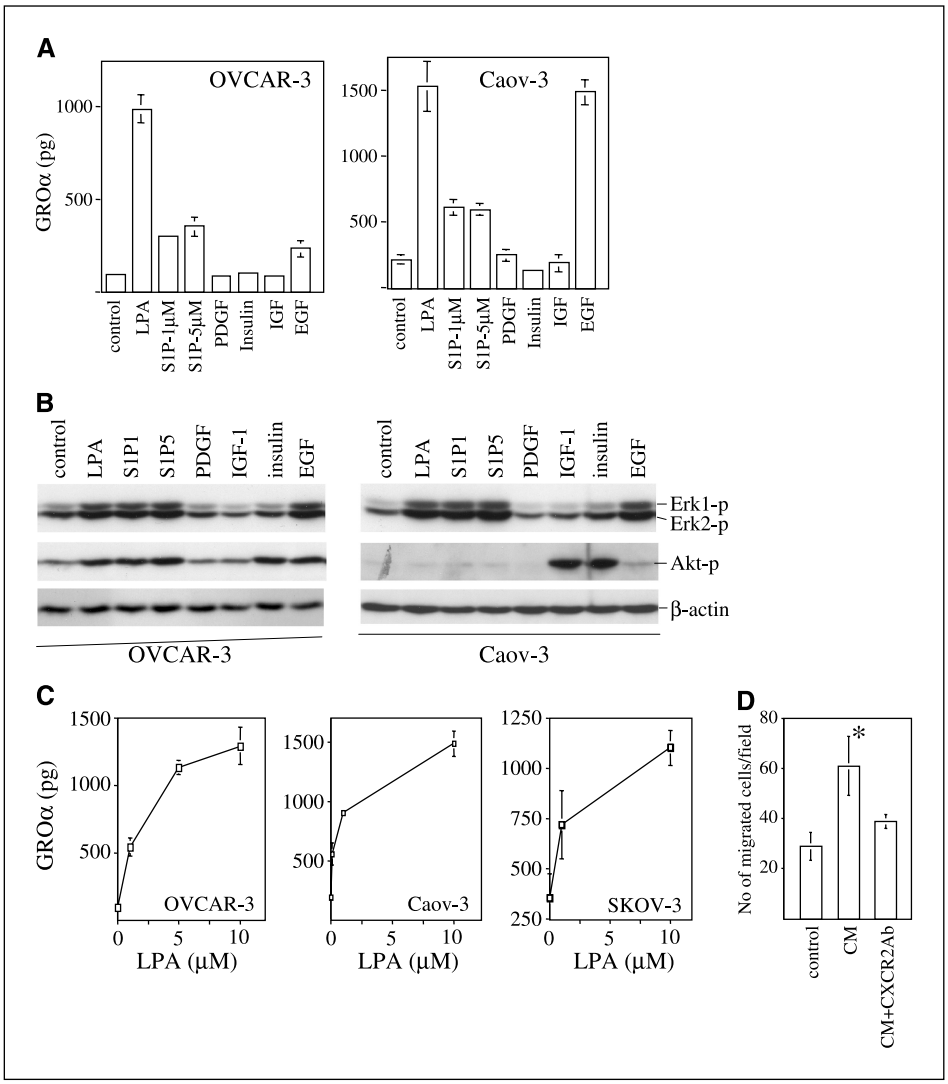

Figure 3. Stimulation of GROα production in ovarian cancer cells by the serum lipid mediators LPA and S1P but not by the serum-containing peptide growth factors PDGF, IGF, or insulin. *A,* OVCAR-3 and Caov-3 cells in six-well plates (5×10^5 per well) were cultured, starved in serum-free medium overnight, and incubated for 16 hours with vehicle (control), LPA (18:1, 10 μmol/L), S1P (1 and 5 μmol/L), PDGF (BB isoform, 50 ng/mL), IGF-I (50 ng/mL), insulin (0.2 μmol/L), or EGF (50 ng/mL). The culture supernatants were collected and analyzed for GROα by ELISA. *B,* OVCAR-3 and Caov-3 cells were stimulated for 10 minutes with various lipid mediators and peptide growth factors at the same concentrations as in (*A*). The stimulated cells were lysed in SDS sample buffer and analyzed by immunoblotting for activation-associated phosphorylation of Erk and Akt using phosphospecific antibodies. *C,* the ovarian cancer cell lines OVCAR-3, Caov-3, and SKOV-3 cells in six-well plates were starved and stimulated for 16 hours with increasing concentrations of LPA. The levels of GROα in culture supernatants were determined by ELISA and the response curves were plotted as amounts of GROα produced by the cells treated with the indicated concentrations of LPA. *D,* chemotactic effect of conditioned medium on HUVEC. Conditioned medium (CM) was collected from Caov-3 cells treated for 20 hours with 10 μmol/L LPA (CM) or with vehicle (*control*). The control medium and CM were added to the bottom of the Boyden chambers. HUVEC (4×10^4 in 100 μL) pretreated for 10 minutes with or without anti-CXCR2 neutralizing antibody (10 μg/mL) were loaded into the upper chambers and incubated at 37°C for 4 hours to allow migration. Each data point is the average number of cells in five randomly selected fields at ×200 magnification. *Columns,* mean of triplicate assays; *bars,* SD. Representative of three independent experiments.

We next examined the effect of LPA and S1P, the two lysophospholipid growth factors present in serum (17, 24), on GROα production. Both LPA and S1P act via G protein–coupled receptors that belong to the endothelial differentiation gene subfamily (24). As described previously, ovarian cancer cells frequently express more than one subtype of LPA and S1P receptors (17, 18, 25).

In OVCAR-3 and Caov-3 cells, LPA strongly stimulated GROα production and accumulation in culture supernatants (Fig. 3*A* and *C*). GROα concentrations in supernatants were increased from 219.2 to 1,538 pg/mL by LPA (10 μmol/L) treatment of Caov-3 cells. Similar increases in GROα production (from 88.2 to 991.6 pg/mL) were observed in OVCAR-3 cells treated with 10 μmol/L LPA. S1P (5 μmol/L) induced increases in GROα levels from 219.2 to 598 pg/mL in Caov-3 cells and from 88.2 to 353.2 pg/mL in OVCAR-3 cells (Fig. 3*A*). When assessed on multiple ovarian cancer cell lines, including SKOV-3 and Dov-13, LPA was consistently more effective than S1P or any peptide growth factors in inducing GROα production, suggesting that LPA is a potent and general regulator of GROα in ovarian cancer cells. Due to the much stronger effect of LPA than EGF, it is unlikely that LPA stimulates GROα production through transactivation of EGF receptor as proposed as mechanisms for other biological actions of LPA (26).

The striking effect of LPA on GROα production in ovarian cancer cells is of particular interest because LPA is present at high levels in ascites of ovarian cancer patients (1-80 μmol/L; refs. 17, 27). As shown in Fig. 3*C*, LPA induced a dose-dependent stimulation of GROα production. The half-maximum concentrations in the ovarian cancer cell lines examined were <1 μmol/L, suggesting that physiologically relevant levels of LPA are sufficient to trigger GROα production. We next examined whether LPA-mediated GROα production in ovarian cancer cells might have a chemotactic effect on endothelial cells, which may contribute to tumor angiogenesis. As shown in Fig. 3*D*, the conditioned medium from Caov-3 cells treated with LPA had an increased ability to induce directional migration of HUVEC compared with the medium collected from vehicle-treated cells. The chemotactic activity was at least partially attributed to GROα present in conditioned medium as incubation of HUVEC with a neutralizing antibody against GROα receptor CXCR2 strongly attenuated the migratory activity.

Transcriptional activation of GROα expression by LPA. To determine whether LPA-enhanced GROα generation in ovarian cancer cells resulted from an increase in GROα mRNA levels, we examined the steady-state levels of GROα transcripts in cells

treated with or without LPA by Northern blot analysis. Incubation of OVCAR-3 cells with LPA led to time- and dose-dependent increase in GROα mRNA (Fig. 4*A*). Peak mRNA levels were observed around 6 hours after addition of LPA (Fig. 4*A*). We also observed a similar pattern of GROα mRNA accumulation in Caov-3 and Dov-13 cells stimulated with LPA (data not shown). We next examined whether the up-regulation of GROα mRNA by LPA was due to an increase in mRNA stability. We took advantage of Dov-13 cells that showed low expression of GROα mRNA in the absence of exogenous LPA, making it possible to compare the stability of GROα mRNA in cells treated with or without LPA (Fig. 4*B*). Following addition of actinomycin D (5 μg/mL) to block new RNA synthesis, GROα transcripts were rapidly degraded in both control and LPA-treated cells (Fig. 4*B*), indicating that the effect of LPA on GROα transcript levels was not caused by changes in the stability of GROα transcripts. Thus, LPA likely increases GROα mRNA levels via transcriptional activation of the GROα promoter. To test this, we transfected ovarian cancer cell lines with pGL2-GROα-Luc, a luciferase reporter construct containing the GROα promoter (−304 to +9; refs. 11, 20). A 6-hour incubation with LPA markedly induced luciferase activity in transfected OVCAR-3 and Caov-3 cells (Fig. 4*C*). Together, the data indicates that LPA induces GROα production, at least in part, through transcriptional activation of the GROα promoter. Deletion analysis revealed that a shorter promoter fragment (−150 to +9) was sufficient to support a full response to LPA (Fig. 4*D*). The 159 bp fragment contains binding sites for Sp1 and nuclear factor-κB, a transcription factor commonly involved in transcriptional activation of cytokines (18). Further deletion of the sequence from the −150 to −84 of the GROα promoter led to >70% loss of the response to LPA (Fig. 4*D*). Thus, the −150 to −84 fragment harbors regulatory element(s) required for LPA-mediated transcription although the exact transcription factors involved are yet to be determined.

Involvement of pertussis toxin–sensitive Gi protein in LPA induction of GROα. To gain insight into the intracellular signaling pathways, we examined the effect of pertussis toxin, a selective inhibitor of Gi proteins, on LPA-mediated GROα production. Preincubation of Caov-3 cells with pertussis toxin dose-dependently inhibited LPA-induced GROα production (Fig. 5). The maximal inhibition observed with 5 to 25 ng/mL pertussis toxin was around 50% (Fig. 5). We observed similar inhibition of LPA-induced GROα production by pertussis toxin in

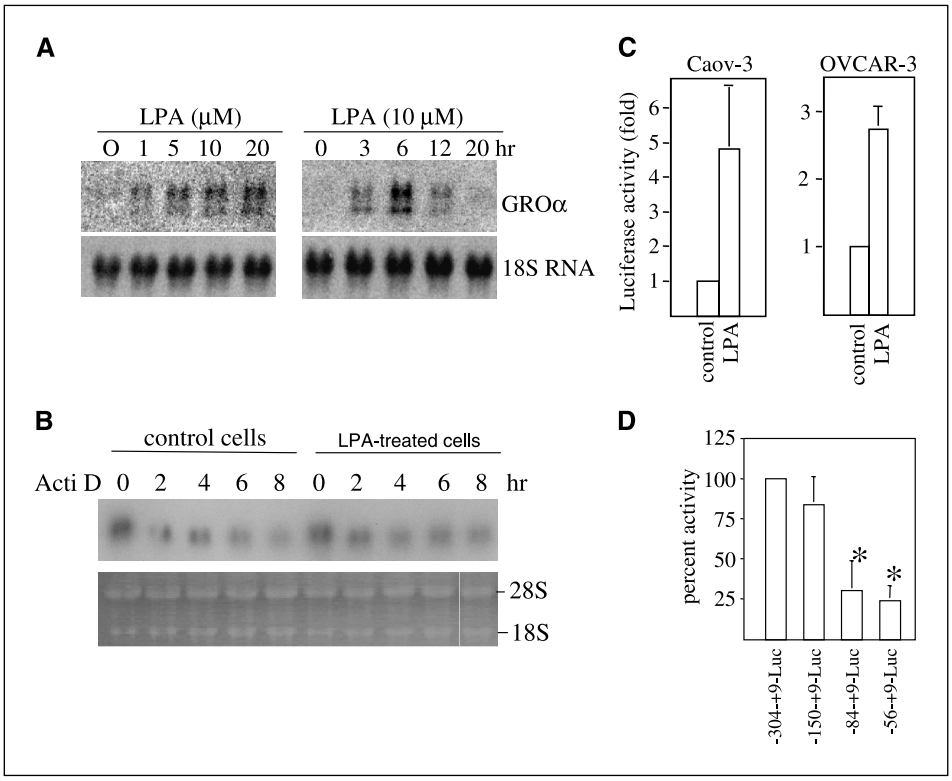

Figure 4. Transcriptional activation of the GROα promoter by LPA. *A,* the induction of GROα mRNA steady-state levels by treatment with LPA. Total cellular RNA was extracted from OVCAR-3 cells treated with LPA (18:1) at indicated concentrations for 3 hours or for the indicated time periods (hours) with 10 μmol/L LPA. The expression of GROα mRNA was examined by Northern blotting (20 μg total RNA) using human GROα cDNA probes. The membrane was rehybridized with a [^{32}P]dCTP-labeled 18S RNA probe to verify equal loading among samples. *B,* analysis of GROα mRNA stability in LPA-treated and untreated Dov-13 cells. The cells were incubated with vehicle (control cells) or 10 μmol/L LPA (LPA-treated cells) for 4 hours before addition of actinomycin D (5 μg/mL) to block new RNA synthesis. Total cellular RNA was extracted at 2, 4, 6, and 8 hours posttreatment with actinomycin D. RNA samples (25 μg for control cells and 15 μg for LPA-treated cells) were analyzed by Northern blotting with ^{32}P-labeled GROα cDNA probes. Ethidium bromide staining of 28S and 18S rRNA on electrophoresis gels was included for comparison of loading. *C,* Luciferase analysis of the GROα promoter. The luciferase construct pGL2-GROα-Luc containing the 313 bp GROα promoter sequence (−304 to +9) was transfected into Caov-3 and OVCAR-3 cells as described in Materials and Methods. Transfected cells were starved and stimulated for 6 hours with vehicle (control) or LPA (18:1, 10 μmol/L) before cell lysates were prepared for measurement of luciferase activity using a luminometer. The activity was normalized on the basis of the activity of cotransfected β-galactosidase driven by the CMV promoter (pCMVβ-gal). *D,* deletion analysis of the GROα promoter. OVCAR-3 cells were transfected with luciferase constructs containing different lengths of the GROα promoter sequences as indicated. LPA-induced increases (fold) in luciferase activity were presented as percentage activity with that in cells transfected with the construct containing the −304 to +9 fragment defined as 100%. *Columns,* mean of triplicate assays; *bars,* SD. Representative of three independent experiments.

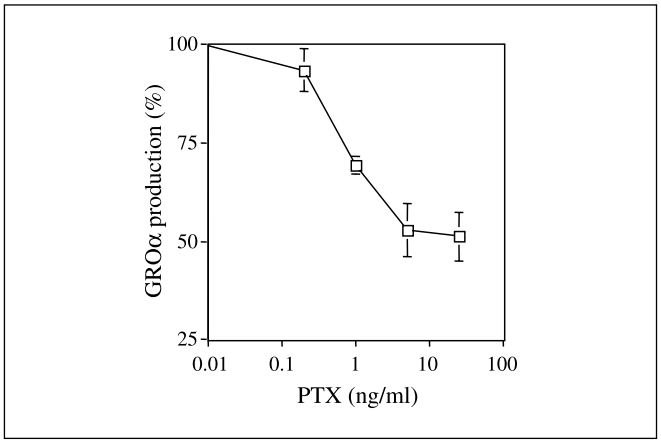

Figure 5. Involvement of pertussis toxin–sensitive Gi protein in LPA stimulation of GROα production. Caov-3 cells in six-well plates (5×10^5 per well) were cultured, starved in serum-free medium for 1 day, and incubated for 16 hours with vehicle or LPA (10 μmol/L) in the presence of indicated concentrations of pertussis toxin (ng/mL). Pertussis toxin was added to culture 8 hours before addition of LPA. GROα levels in culture supernatants were determined by ELISA. LPA-induced net increases in GROα concentrations were presented as percentage activity with the increases induced by LPA in the absence of pertussis toxin defined as 100%.

OVCAR-3 cells (data not shown), indicating the involvement of both Gi-dependent and Gi-independent mechanisms in LPA activation of GROα expression.

LPA receptor–dependent induction of GROα. We next examined whether the effect of LPA on GROα production is mediated by LPA receptors. The ability of LPA to induce GROα production is not restricted to ovarian cancer cells. LPA also induced GROα generation in the breast cancer cell lines MCF-7 and MDA-MB-468. The induction of GROα by LPA in these breast cancer cell lines was not as efficient as in ovarian cancer cells mainly due to the heavy basal levels of GROα in unstimulated MCF-7 and MDA-MB-468 cells (data not shown). MDA-MB-468 expresses one or more isotypes of LPA receptors as others and we described previously (18, 28). In contrast to these responsive breast cancer cell lines, LPA did not induce GROα production in SKBr-3 cells (Fig. 6*B*), which do not express detectable levels of LPA_1, LPA_2, or LPA_3 receptor mRNA as assessed by Northern blot analysis (Fig. 6*A*). The lack of functional LPA receptors in SKBr-3 cells has been described previously (18, 28).

To determine whether ectopic expression of LPA receptors could restore GROα production in response to LPA, SKBr-3 cells were transiently transfected with LPA receptors, LPA_1, LPA_2 and LPA_3, each tagged with the Flag M2 at the NH_2 terminus for detection of expression (18). Expression of each LPA receptor in SKBr-3 cells dramatically increased LPA-induced GROα production (Fig. 6*C*) although transient transfection efficiency in SKBr-3 cells was generally below 20% as we described previously (18). Interestingly, transfection of the LPA_2 receptor consistently led to GROα production to levels greater than transfection of the LPA_1 or LPA_3 receptor. The obviously stronger effect of LPA_2 on LPA-induced GROα production suggests that LPA_2 may function as a primary LPA receptor accounting for GROα production although other LPA receptors, when overexpressed, also have the ability to mediate GROα generation. We have shown previously that LPA_2 is overexpressed in primary ovarian cancers and its expression is detectable in most ovarian cancer cell lines (17, 18), potentially contributing to the elevated levels of GROα observed in ovarian cancer patients.

Certain species of LPA, such as 14:0 LPA, show a selective ability to activate the LPA_2 receptor over other receptor subtypes (29). If the LPA_2 receptor is indeed physiologically more critical than LPA_1 and LPA_3 in connection to GROα production, challenging cells with the LPA_2-selective agonist 14:0 LPA might lead to more effective induction of GROα than the pan-receptor agonist 18:1 LPA. To examine this hypothesis, we compared the ability of 14:0 and 18:1 LPA to induce GROα production in OVCAR-3 cells that endogenously express all three LPA receptors (Fig. 6*A*). Dose-response curves show that 14:0 LPA was indeed more effective than 18:1 LPA in inducing GROα production (Fig. 7), further highlighting the importance of the LPA_2 receptor in activation of GROα expression.

Inhibition of LPA- and FBS-induced GROα production by down-regulating the LPA_2 receptor and by the LPA receptor antagonist Ki16425. The observation that the LPA_2 receptor was more effective than LPA_1 and LPA_3 in reconstituting LPA-dependent GROα production prompted us to analyze whether endogenous expression of LPA_2 is necessary for LPA-triggered GROα production in ovarian cancer cells. To this end, we used LPA_2-specific siRNA to suppress LPA_2 expression in OVCAR-3 cells. The efficient inhibition of LPA_2 expression was verified by real-time reverse transcription-PCR (RT-PCR) analysis of the cells transfected with LPA_2-specific siRNA and those transfected with nontarget control siRNA. As shown in Fig. 8*A*, transfection with LPA_2-specific siRNA led to >80% decrease in LPA_2 mRNA levels. This down-regulation of LPA_2 expression was accompanied by >70% inhibition in LPA-induced GROα production, indicating a major input from the LPA_2 receptor in mediation of GROα production by LPA (Fig. 8*A*). Interestingly, siRNA down-regulation of LPA_2 also significantly diminished FBS-afforded GROα production by ~40% (Fig. 8*A*). Although the inhibition of FBS-mediated GROα production by LPA_2 siRNA was not as dramatic as seen with the effect of LPA, the results suggest that FBS drives GROα production at least partially through its bioactive constituent LPA interacting with the LPA_2 receptor.

To further confirm the direct role of LPA receptors, we assessed the effect of Ki16425, a recently developed LPA receptor antagonist (30), on LPA-induced GROα generation in OVCAR-3 cells that express $LPA_{1\text{-}3}$ receptors (Fig. 6*A*). Ki16425 is a relatively broad inhibitor for each of the three LPA receptor subtypes with the potency order of $LPA_1 > LPA_3 \gg LPA_2$ (30). Indeed, LPA-induced GROα production was sensitive to Ki16425 in OVCAR-3 cells (Fig. 8*B*). Interestingly, the curve of Ki16425 inhibition of LPA-induced GROα production was compatible with the involvement of two types of LPA receptors of different sensitivity to Ki16425 (Fig. 8*B*). The first, likely representing LPA1 and/or LPA_3, was blocked by ≤5 μmol/L Ki16425 and the other, likely LPA_2, was affected only by 10 to 20 μmol/L Ki16425. Approximately 20% of LPA stimulatory activity remained when 20 μmol/L of Ki16425 was present, consistent with the requirement of high concentrations of Ki16425 for antagonizing LPA_2.

Discussion

Although expression of GROα is associated with tumor growth, angiogenesis, and metastasis of a variety of human cancers (11–16, 22), the regulatory mechanism behind the deregulated expression of the GROα gene in transformed cells is poorly

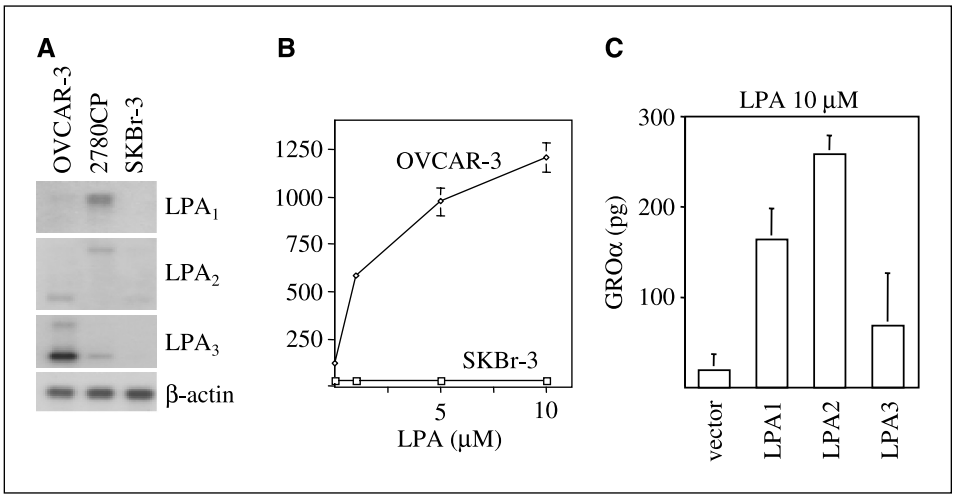

Figure 6. Reconstitution of LPA-induced GROα production in nonresponsive SKBr-3 cells by transfection with LPA receptors. *A,* lack of significant expression of LPA receptors (LPA_1, LPA_2, and LPA_3) in SKBr-3 cells. Total cellular RNA (20 μg) from OVCAR-3, 2780CP, and SKBr-3 cells was analyzed by Northern blot analysis with ^{32}P-labeled human LPA_1, LPA_2, and LPA_3 cDNA as probes. Reprobing with β-actin cDNA probe was included to show equal loading among samples. *B,* lack of response to LPA in SKBr-3 cells. SKBr-3 and the responsive OVCAR-3 cells in six-well plates (5×10^5 per well) were starved and incubated for 16 hours with LPA at indicated concentrations. The conditioned medium was collected and GROα levels were determined by ELISA. *C,* restoration of LPA response in SKBr-3 cells by ectopic expression of LPA receptors. SKBr-3 cells were transfected with an empty vector or with an LPA_1, LPA_2, and LPA_3 expression vector. The transfected cells were starved and stimulated with vehicle or 10 μmol/L LPA for 24 hours. GROα production was determined by ELISA analysis of the culture supernatants. Net increases in GROα production induced by LPA were calculated by subtracting background in vehicle-treated cells from the levels of the corresponding LPA-treated cells. *Columns,* mean of triplicates; *bars,* SD. Representative of three independent experiments.

understood. In the present study, we showed that LPA, acting primarily on the LPA_2 receptor, promotes GROα production in ovarian cancer cells through a transcriptional mechanism. This likely contributes to the elevated levels of GROα in the plasma and to the concentration gradient formed between ascites and plasma of ovarian cancer patients. Our finding is of particular interest as LPA has been identified as an important growth factor for ovarian cancer (17, 31). Its concentration is markedly and consistently elevated in the ascites of ovarian cancer patients, ranging from 1 to 80 μmol/L (17, 27, 31), providing an LPA-rich microenvironment for ovarian tumor cells. Thus, it is conceivable that GROα production in ovarian cancer patients is an outcome of LPA interaction with ovarian tumor cells in ascites.

LPA, as an extracellular mediator, stimulates growth, survival, and motility of ovarian cancer cells (17, 31). Recent studies from

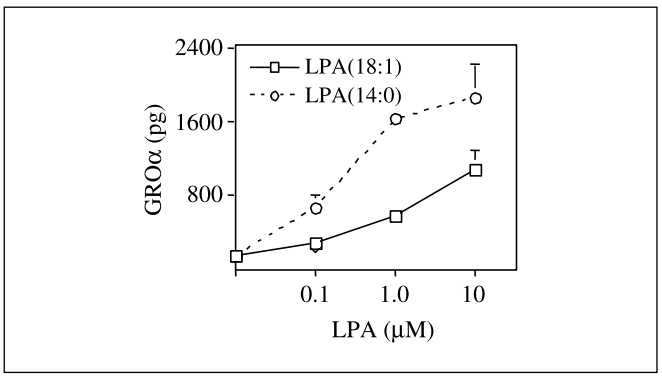

Figure 7. More potent induction of GROα production by 14:0 LPA, an LPA_2-seletive agonist, than the generic agonist 18:1 LPA. OVCAR-3 cells in six-well plates (5×10^5 per well) were starved overnight and stimulated with 14:0 or 18:1 LPA for 16 hours at indicated concentrations. The amounts of GROα present in culture supernatants of the stimulated cells were quantified by ELISA. *Points,* mean of triplicates; *bars,* SD. Representative of two independent experiments.

our group and from other laboratories indicate that LPA may mediate oncogenesis through regulation of gene expression. Vascular endothelial growth factor (VEGF; ref. 32), urokinase-type plasminogen activator (uPA; refs. 33, 34), interleukin (IL)-6 (18), IL-8 (18, 35), and Cox-2 (36) have been identified as LPA-regulated genes. LPA may contribute to cancer development or progression at least partially through up-regulation of a suite of oncoproteins. Our current study identifies GROα as a novel target gene of LPA in ovarian cancer cells. Previous studies of GROα in other human malignancies indicate that GROα may play a role in multiple carcinogenic processes. In squamous cell carcinoma and oral cancer, continuous expression of GROα correlates with tumor microvessel density (12, 13, 16), consistent with GROα functioning as chemokine angiogenic factor. In these models, the expression of GROα is also associated with leukocyte infiltration and increased lymph node metastasis in a host CXCR2-dependent fashion (13, 16). In the present study, we also showed that GROα accumulated in conditioned medium of ovarian cancer cells treated with LPA could trigger chemotactic response in human endothelial cells. In addition to this paracrine mechanism, GROα acts as an autocrine growth factor and stimulates proliferation and invasiveness of melanoma cells and colon carcinoma cells that express the CXCR2 receptor for GROα (4–8, 14).

We previously showed that primary ovarian cancers and established ovarian cancer cell lines overexpress the LPA_2 receptor (17, 18), which is consistent with a critical role for this receptor in GROα production in ovarian cancer cells. In addition to ovarian cancer, the LPA_2 receptor is also overexpressed in colon cancer and colorectal cancer (37, 38), differentiated thyroid cancer (39), and invasive ductal carcinomas (40). Therefore, amplification of LPA signaling through overexpression of specific LPA receptors could contribute to the pathogenesis of many types of human malignancies. During the current study, we have also observed that LPA induced GROα production by breast cancer cell lines. Because of the widespread expression of LPA receptors and the presence of

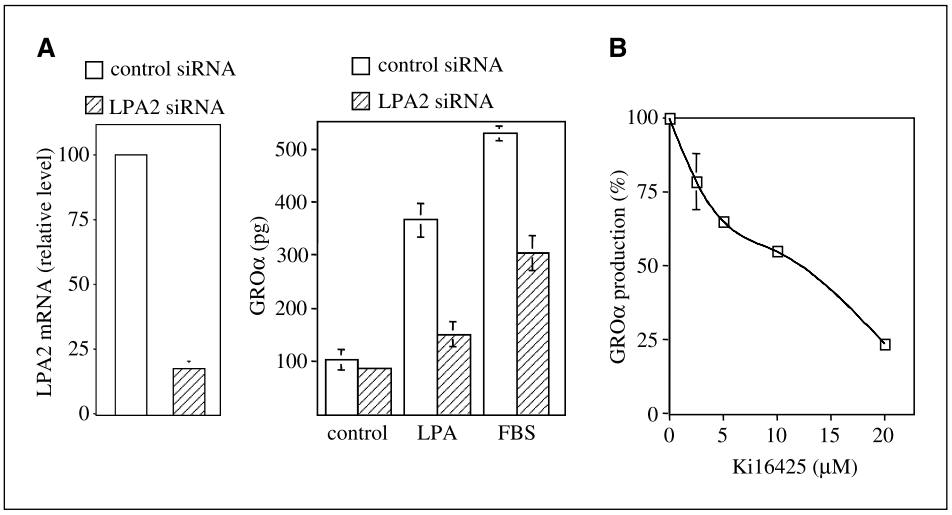

Figure 8. Inhibition of LPA-induced GROα production by siRNA down-regulation of LPA_2 expression and by the LPA receptor antagonist Ki16425. *A,* OVCAR-3 cells were plated in 24-well plates (1.2×10^5 per well) in complete medium free of antibiotics. Next day, the cells were transfected with LPA_2-specific siRNA or nontarget control siRNA using LipotectAMINE 2000 as described in Materials and Methods. Approximately 36 hours after transfection, parts of the transfected cells were used for isolation of total cellular RNA followed by quantitative RT-PCR analysis of LPA_2 mRNA level (*left*). The remaining cells in 24-well plates were starved and stimulated with 10 μmol/L LPA or 5% FBS for 16 hours. GROα produced in culture supernatants were determined by ELISA (*right*). *B,* OVCAR-3 cells in six-well plates (5×10^5 per well) were cultured, starved in serum-free medium overnight, and incubated for 16 hours with vehicle or LPA (10 μmol/L) in the presence of indicated concentrations of Ki16425 (μmol/L). Ki16425 was added to culture 45 minutes before addition of LPA. GROα levels in culture supernatants were determined by ELISA. LPA-induced net increases in GROα concentrations were presented as percentage activity with the increase induced by LPA in the absence of Ki16425 defined as 100%. *Points,* mean of triplicates; *bars,* SD. Representative of three independent experiments.

LPA in serum and other biological fluids, LPA induction of GROα expression likely represents a general mechanism for deregulated expression of this cytokine in various human malignancies.

In addition to the role in the regulation of GROα expression, we previously showed that LPA_2 was the most intimately involved in LPA-dependent IL-6 and IL-8 production in ovarian and breast cancer cells although other LPA receptors could also mediate the response when heterologously overexpressed (18). Similarly, Hu et al. (32) described the correlation of the LPA_2 receptor, rather than LPA_1 or LPA_3, with the ability of LPA to induce VEGF expression in ovarian cancer cells. The importance of LPA_2 in modulation of gene expression is further highlighted by the observation that transgenic expression of LPA_2 driven by an ovary-selective promoter led to the production of higher levels of VEGF and uPA mRNA and proteins in the ovaries of transgenic mice (41). These studies together suggest that LPA_2, an LPA receptor subtype frequently overexpressed in multiple types of human cancers, plays a critical role in LPA regulation of expression of cytokines and angiogenic, invasive or metastatic factors.

Acknowledgments

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