Mechanisms for lysophosphatidic acid-induced cytokine production in ovarian cancer cells

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Running Title: LPA-induced cytokine production
SUMMARY

A potential role for lysophosphatidic acid (LPA) in human oncogenesis was first suggested by the observation that LPA is present at elevated levels in ascites of ovarian cancer patients. In the current study, we demonstrated that LPA is a potent inducer of interleukin-6 (IL-6) and interleukin-8 (IL-8) production in ovarian cancer cells. Both IL-6 and IL-8 have been implicated in ovarian cancer progression. We characterized the IL-8 gene promoter to ascertain the transcriptional mechanism underlying LPA-induced expression of these cytokines. LPA stimulated the transcriptional activity of the IL-8 gene with little effect on IL-8 mRNA stability. The optimal response of the IL-8 gene promoter to LPA relied on binding sites for NF-κB and AP-1, two transcription factors that were strongly activated by LPA in ovarian cancer cell lines. Positive regulators of the NF-κB and AP-1 pathways synergistically activated the IL-8 gene promoter. Further, the effect of LPA on IL-6 and IL-8 generation is mediated by the Edg LPA receptors as enforced expression of LPA receptors restored LPA-induced IL-6 and IL-8 production in non-responsive cells and enhanced the sensitivity to LPA in responsive cell lines. The LPA$_2$ receptor was identified to be the most efficient in linking LPA to IL-6 and IL-8 production although LPA$_1$ and LPA$_3$ were also capable of increasing the response to a certain degree. These studies elucidate the transcriptional mechanism and the Edg LPA receptors involved in LPA-induced IL-6 and IL-8 production and suggest potential strategies to restrain the expression of these cytokines in ovarian cancer.
INTRODUCTION

Lysophosphatidic acid (LPA), a naturally occurring phospholipid, activates distinct members of the endothelial differentiation gene (Edg) subfamily of G protein-coupled receptors to elicit multiple cellular responses (1). LPA was first implicated in human carcinogenesis by the observation that LPA is present at elevated levels in ascites of ovarian cancer patients (2-5). In culture, LPA exhibits multiple cellular effects on ovarian carcinoma cell lines including stimulation of cell proliferation (6, 7), promotion of cell survival and drug resistance (8, 9), and induction of cell motility and invasion (10-11). However, the exact role of LPA in human oncogenesis, particularly in vivo, remains poorly understood.

It has been recently reported that LPA and other phospholipids stimulate expression of interleukin-8 (IL-8) in ovarian cancer cell lines (12). Interestingly, in ovarian cancer patients, serum levels of IL-8 and interleukin-6 (IL-6) are elevated (13-15). The concentrations of IL-8 and IL-6 are several magnitudes higher in ascitic fluid than in serum (13), implying that IL-8 and IL-6 are produced in ascites, probably by cancer cells, and migrate from the peritoneal cavity to the circulation similar to other tumor markers such as CA125 (16). Further, high concentrations of IL-8 and IL-6 in ovarian cancer correlate with poor initial response to chemotherapy and with poor prognosis, respectively (13). In animal models, expression of IL-8 and other growth factors such as vascular endothelial growth factor (VEGF) is associated with increased tumorigenicity, ascites formation, angiogenesis and invasiveness of ovarian cancer cells (17, 18). Development of the drug resistant phenotype in ovarian cancer cell lines is accompanied by overexpression of IL-8 and IL-6 (19, 20). Taken together, these studies suggest that IL-8 and IL-6 are important modulators of cancer progression, drug resistance and prognosis in ovarian cancer.
The prominent ability of LPA to stimulate expression of IL-8 in ovarian cancer cells highlights the possibility that elevated levels of IL-8 seen in ovarian cancer patients are attributable to LPA that co-localizes with tumor cells in the ascitic fluid of ovarian cancer patients (3-5). Therefore, it is of particular interest to investigate the mechanism by which LPA induces IL-8 expression in ovarian cancer cells. In the current study, we showed that LPA induced expression of both IL-8 and IL-6 in ovarian cancer cells. LPA stimulated increases in mRNA levels of these cytokines due to transcriptional activation with limited increases in mRNA stability. We characterized the IL-8 gene promoter (21, 22) by luciferase assays and identified a fragment 133 bp upstream of the transcription initiation site responsible for the full response to LPA. The region harbors binding sites for the transcription factors AP-1, NF-IL-6 and NF-κB (21-23). Deletion and mutation analyses of the sequence indicated that the optimal response to LPA relied on binding sites for NF-κB and AP-1. In ovarian cancer cell lines, LPA potently stimulated NF-κB and AP-1 transcriptional activity as determined by luciferase assays using synthetic NF-κB or AP-1-responsive promoters. Co-expression of positive regulators of the NF-κB and AP-1 transcription factors was sufficient to stimulate the transcriptional activity of the IL-8 gene promoter in ovarian cancer cells. Further, the effect of LPA on IL-6 and IL-8 expression is mediated through the Edg LPA receptors (24-26). The LPA₂ receptor was found to be the most efficient in coupling LPA to IL-6 and IL-8 production although LPA₁ and LPA₃ could also mediate the response to a certain degree.

**EXPERIMENTAL PROCEDURES**

**Reagents**

LPA (myristoyl, palmitoyl and oleoyl) and phosphatidic acid (PA, dipalmitoyl) were purchased from Avanti Polar Lipids (Alabaster, AL). Before use, these phospholipids were dissolved in PBS containing 1% fatty acid-free bovine serum albumin (BSA) (Roche Molecular Biochemicals). Platelet-derived growth
factor (PDGF), epidermal growth factor (EGF), fetal bovine serum (FBS), actinomycin D and anti-flag M2 and anti-β-actin monoclonal antibodies were obtained from Sigma. Insulin-like growth factor 1 (IGF-1) was from Upstate Biotechnology (Lake Placid, NY). Anti-phospho Erk antibody and luciferase reaction kits were purchased from Promega. Anti-phospho Akt (Ser-473) antibody was from Cell Signaling. Anti-V5 monoclonal antibody was obtained from Invitrogen (Carlsbad, CA). The pharmacological inhibitors of signal transduction pathways (PD98059, SP600125, SB203580 and wortmannin) were purchased from Calbiochem (San Diego, CA).

Cells

The source and maintenance of ovarian cancer cell lines including OVCAR-3, SKOV-3, Dov-13, HEY, OVCA432, Caov-3 and A2780CP have been described previously (27). The human breast carcinoma cell lines BT549 and SKBr-3 were obtained from ATCC. MDA468 was provided by Dr. Y Yu (MD 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 less than 10 weeks in continuous culture.

Measurement of IL6 and IL-8 production by ELISA

Culture supernatants of cell lines treated without or with LPA or other ligands were collected and analyzed for IL-6 and IL-8 concentrations by ELISA using the Quantikine IL-6 or IL-8 ELISA kits (R&D Systems). Concentrations of IL-6 and IL-8 in the supernatants were calculated by comparing the absorbance of samples to standard curves.

Western blot

Cells were lysed in ice-cold X-100 lysis buffer [1% Triton X-100, 50 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 % glycerol, 100 mM NaF, 10 mM Na pyrophosphate and proteinase inhibitor mixture (Roche)]. Total cellular protein was resolved by SDS/PAGE, transferred to immobilon [poly(vinylidene difluoride)], and immunoblotted with antibodies following the protocols provided by manufacturers. Immunocomplexes were visualized with an enhanced chemiluminescence
detection kit (Amersham Pharmacia) using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA).

**Northern Blot Analysis**

Total cellular RNA and PolyA⁺ RNA were extracted from cultured cells using the TRIzol Reagent and the FastTrack 2.0 mRNA Isolation kit, respectively, following the instructions of the supplier (Invitrogen). 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 crosslinking, and then prehybridized and hybridized to ³²P-labeled cDNA probes as described previously (27). The human LPA₁, LPA₂ and LPA₃ cDNAs were isolated from expression vectors. The cDNA clones for the human *IL-6* (28) and *IL-8* (29) genes were from ATCC. Quality of RNA samples was confirmed by re-hybridization to the human glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) cDNA or to the DNA of 18S rRNA obtained from the ATCC.

**mRNA stability assay**

DOV-13 cells were grown to subconfluence and starved for 14 hours. The cells were then stimulated for 4 hours in serum-free medium with or without 10 μM LPA before addition of actinomycin D (5 μg/ml). Total cellular RNA was prepared at 0, 2, 4, 6, and 8 h after addition of actinomycin D and examined by Northern blot analysis as described above. The intensity of *IL-8* mRNA hybridization bands was quantified by densitometry.

**Plasmids, transfection and luciferase assays**

The LPA₁ receptor expression vector, pcDNA3-Flag-LPA₁, (30) was provided by Dr. WH Moolenaar (The Netherlands Cancer Institute, Amsterdam, the Netherlands). LPA₂ and LPA₃ cDNAs were obtained from OVCAR-3 cells by RT-PCR and inserted into pcDNA3 that had been pre-modified to contain the Flag M2 DNA sequence located at the 5’-terminal of genes to be inserted. The structures of pcDNA-
Flag-LPA1, pcDNA3Flag-LPA2 and pcDNA3Flag-LPA3 vectors were confirmed by automatic sequencing. The luciferase vectors containing various lengths or mutants of the IL-8 promoter (22, 23) were gifts of Dr. IJ Fidler (MD Anderson Cancer Center). The NF-κB-responsive luciferase vector pNF-κB-Luc was purchased from Stratagene (La Jolla, CA). The AP-1-responsive luciferase vector p3xTRE-TK-Luc was constructed as we described previously (31). To generate mammalian expression vectors for c-Jun and RelA/p65, the mouse c-Jun cDNA (32) (obtained by RT-PCR from Swiss 3T3 cells) and the human RelA/p65 cDNA (33) (obtained from Dr. P Chiao, MD Anderson Cancer Center) were placed under the control of the CMV promoter of the pcDNA3 expression vector.

For luciferase assays, ovarian cancer cell lines were transfected with luciferase reporter constructs using Fugene 6 according to the instructions of the manufacturer (Roche). About 60 hours after transfection, cells were starved for 48 hours before stimulation with LPA or vehicle for 6 hours. Cell extracts were prepared and assayed for luciferase activity using a kit from Promega. The luciferase activity was normalized on the basis of the activity of cotransfected β-galactosidase reporter driven by the CMV promoter (pCMVβ-gal). Alternatively, fold increases in luciferase activity were normalized by subtracting LPA-induced non-specific activity increases in cells transfected with pGL2-Luc in which the luciferase reporter is driven by the constitutive SV40 promoter.

**Restoration of LPA-induced IL-6 and IL-8 production in SKBr-3**

To determine whether enforced expression of LPA receptors can restore cytokine production in response to LPA, the non-responsive 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, cells were serum starved for 36 hours prior to stimulation with LPA for 24 hours. The conditioned medium was harvested for ELISA analysis of IL-6 and IL-8. In addition, SKBr-3 cells were co-transfected with each LPA receptor along with a GFP vector (pEGFP, BD Biosciences, San Jose, CA). Two days after transfection, cells expressing high levels of GFP were isolated by FACS
(FACScalibur; Becton Dickinson, Mountain View, CA) and replated. Subsequent examination under fluorescence microscope indicated that >90% of the population was GFP positive. These cells were starved and stimulated with LPA to determine the effect of LPA on Erk phosphorylation in SKBr-3 cells expressing exogenous LPA receptors.

**Immunofluorescent staining**

Transfected cells were grown on cover slips. Before staining, cells on the cover slips were washed with PBS and fixed with 4 % paraformaldehyde. Cells were then blocked with 5 % goat serum/0.1 % Triton X-100/PBS for 1 hour prior to incubation with anti-Flag M2 monoclonal antibody (Sigma, 4.5 μg/ml/0.5% goat serum/0.1% X-100/PBS) for 1 hour. FITC-conjugated goat anti-mouse Ig G (DAKO Corp. Carpinteria, CA) was used as secondary antibody (1:140 in 0.5 % goat serum/0.1% Triton X-100/PBS, 1 hour). Percentages of positive cells were determined by counting more than 500 cells under fluorescent microscope.

**Generation of lentivirus LPA receptor constructs and infection of cells**

The ViralPower™ Lentiviral Expression System was obtained from Invitrogen. The system allows creation of a replication-incompetent, HIV-1-based lentivirus that can efficiently transduce mammalian cell lines (34,35). The cDNA of LPA1, LPA2 and LPA3 was amplified by PCR from their pcDNA3-based expression vectors. The PCR products were cloned into a Gateway entry vector (pENTR4) (Invitrogen) using Nco I and Xho I restriction sites. The viral constructs were made through homologous recombination between pENTR4-LPA1-3 and the lentiviral destination vector pLenti6/V5-DEST. In the resulting viral constructs, each of LPA receptors was tagged with V5 at the C-terminal. The downstream anti-blasticidin gene allows selection of transduced cells. The structures of the pLenti6-LPA1, pLenti6-LPA2 and plenti6-LPA3 as well as the control vector pLenti6-LacZ (provided by the Invitrogen kit) were confirmed by automatic sequencing.
To generate high titer of viral stocks, 293FT cells were co-transfected (Lipofectamine™ 2000) with the pLenti6-LPA vector (3 μg) and a mix (9 μg) of the three packaging plasmids (pLP1, pLP2 and pLP/VSVG) which supply \textit{in trans} the structural and replication proteins required for production of the lentivirus. Approximately 72 hours after the beginning of transfection, the supernatants were harvested, cleared by brief spin and stored at −80°C.

Ovarian cancer cell lines in 6-well or 12-well plates at around 50% confluence were incubated for 18 hours with 1 ml or 0.75 ml of undiluted viral supernatants in the presence of Polybrene (7.5 μg/ml). The infected cells were then kept in regular complete medium for 48 hours. Before stimulation with LPA, the cells were starved overnight in serum-free medium. High efficiency of gene transduction by lentivirus in SKOV-3 cells was suggested by the resistance of the majority of the cells to blasticidin selection and the presence of high percentages of cells (>70%) stained positive for β-galactosidase activity in the pLenti6-LacZ virus-infected cells.

\textbf{Statistics}

The significance of the results of luciferase assays was determined using the Student’s \textit{t} test.

\textbf{RESULTS}

\textbf{LPA induces production of IL-6 and IL-8 in ovarian cancer cells}

Recent studies including our own indicate that, in ovarian cancer cells, LPA induces expression of certain genes, such as VEGF (36), urokinase plasminogen activator (uPA) (37) and IL-8 (12) that are involved in angiogenic and metastatic processes in human cancer. These studies suggest that LPA may contribute to cancer progression through regulation of gene expression. As IL-8 and its related pro-inflammatory molecule IL-6 are important mediators of ovarian cancer (13-15, 17-20), we focused on the effect of LPA on the expression of these two molecules. In a number of ovarian carcinoma cell lines including OVCAR-3, SKOV-3 and DOV-13, LPA stimulates production of not only IL-8 but also IL-6 in a
dose-dependent manner (Fig. 1). Overnight incubation of OVCAR-3 cells with 10 μM of LPA led to 50-100 fold increases in IL-6 and IL-8 concentrations in the culture supernatants as analyzed by ELISA. Since Dov-13 cells produce high basal levels of IL-6 and IL-8, fold increases in response to LPA is less dramatic than those observed in OVCAR-3 and SKOV-3 cells. However, LPA-treated Dov-13 cells generated large quantities of IL-6 and IL-8 (nanograms/million cells). In OVCAR-3 and Dov-13 cells, the half maximal effect dose of LPA is less than 1 μM (Fig. 1), suggesting the involvement of high-affinity LPA receptors.

LPA induced IL-6 and IL-8 generation in other ovarian cancer cell lines we evaluated (HEY, OVCA432, Caov-3 and A2780CP). In contrast, the breast cancer cell line SKBr-3 had minimal IL-6 and IL-8 production in response to LPA (Fig. 1). This, however, does not necessarily reflect a differential response to LPA between breast and ovarian cancer cells as other breast cancer cell lines such as BT549 and MDA468 do respond to LPA with IL-6 and IL-8 production (data not shown).

We then compared the effects of different species of LPA, FBS and peptide growth factors on IL-6 and IL-8 production in OVCAR-3 cells (Fig. 2A). At 10 μM, myristoyl (14:0) and oleoyl (18:1) LPA are more potent than palmitoyl LPA (16:0) in stimulating IL-6 and IL-8 expression. Phosphatidic acid (PA, 16:0) demonstrated a much weaker stimulatory effect on IL-6 and IL-8 production (Fig. 2A). EGF had only marginal activity compared to LPA. In OVCAR-3 cells, the EGF receptor is expressed and functional as reflected by EGF-mediated activation of Erk and Akt (Fig. 2B). FBS contains multiple growth factors including LPA (38-40). Similar to LPA, FBS strongly induced IL-6 and IL-8 expression (Fig. 2A).

However, peptide growth factors present in FBS, such as IGF-1 (Fig. 2A) and PDGF (data not shown), did not promote production of these cytokines by OVCAR-3 cells. OVCAR-3 cells lack functional receptors for IGF-1 and PDGF as suggested by the absence of signaling responses, such as activation of Erk and Akt, to IGF-1 and PDGF (Fig. 2B). SKOV-3 cells are also devoid of functional responses to IGF-1 and PDGF (data not shown). In human and murine fibroblast lines, IGF-1 and PDGF strongly activated Erk and Akt (data not shown), indicating that these peptide growth factors we used were biologically active. These
observations suggest that the stimulation of IL-6 and IL-8 production in ovarian cancer cells is unique to LPA and is apparently not conferred by transactivation of EGF or PDGF receptors as proposed as mechanisms for other biological actions of LPA (41, 42).

**LPA stimulates IL-8 production mainly through activation of transcription**

Northern blotting analyses confirmed that incubation of ovarian cancer cell lines with LPA led to marked increases in steady-state levels of IL-8 mRNA and IL-6 mRNA (data not shown). We next examined whether the up-regulation of IL-8 mRNA by LPA is due to an increase in mRNA stability. We measured IL-8 mRNA stability in Dov-13 cells treated with or without LPA. This cell line showed modest levels of constitutive expression of IL-8 mRNA under serum-starved, unstimulated conditions, making it possible to compare mRNA stability in LPA-treated and untreated conditions. After addition of actinomycin D to stop new RNA synthesis (23), IL-8 transcripts were rapidly degraded in both LPA-treated and control cells (Fig. 3A). The degradation curves show only a slight increase, if any, in the stability of IL-8 transcripts in LPA-treated cells (Fig. 3A). The results suggest that LPA stimulates expression of IL-8 mRNA mainly through transcriptional activation although this does not exclude the possibility that the minor increase in transcript stability may contribute modestly to the effect of LPA on IL-8 mRNA levels.

**LPA activates the IL-8 gene promoter**

To confirm that LPA induces transcriptional activation of the IL-8 gene, we characterized the IL-8 gene promoter (21, 22) by luciferase assays. Ovarian cancer cell lines were transfected with the plasmid pIL-8-Luc in which the luciferase reporter gene is placed under the control of the 5’-flanking region spanning from -1481 to +44 bp of the *IL-8* gene (22). In all ovarian cancer cell lines tested, treatment of transfected cells with LPA resulted in marked increases in luciferase activity (Fig. 3B), confirming that LPA indeed triggers transcriptional activation of the *IL-8* gene promoter. In
contrast, incubation with LPA only slightly stimulated transcriptional activity of the SV40 promoter as reflected by a limited increase in luciferase activity in OVCAR-3 cells transfected with pGL2-Luc (Fig. 3B).

We then examined the responses of a series of deletion mutants of the IL-8 gene promoter to LPA in OVCAR-3 cells. As illustrated in Fig. 4A, the -133– +44 proximal fragment was identified to be the minimum sequence that retained the full response to LPA. This region of the promoter harbors three well-defined transcription factor-binding sites: AP-1, NF-IL-6 and NF-κB. Further deletion of the -133– -98 region containing the AP-1 binding site caused an approximately 60 % decrease in luciferase activity, indicating requirement of this part of the IL-8 promoter, probably the AP-1 binding site, for the full response to LPA. Additional deletion of the -98– -85 sequence which contains the NF-IL-6 binding site further reduced the response to LPA by about 30% (Fig. 4A). The results indicate that the sequence from –133 to –85 is required for LPA-induced transcriptional activation of the IL-8 gene.

To further characterize the -133– +44 fragment of the IL-8 gene promoter, we tested a number of luciferase constructs with specific mutations in each of the transcription factor-binding sites (AP-1, NF-IL-6 or NF-κB) (Fig. 4B). The point mutations made in each of the cis elements eliminate the ability to bind to their corresponding transcription factors (22, 23). Consistent with the deletion analysis, mutation of the AP-1 binding site resulted in an approximately 50 % decrease in luciferase activity. Mutation of the NF-IL-6 site led to a small decrease in LPA-stimulated luciferase activity that was not statistically significant. The most dramatic effect was observed with mutation of the NF-κB binding site that caused loss of nearly 90% of LPA-induced luciferase activity (Fig. 4B), indicating that the NF-κB site is obligatory for LPA-conferred activation of the IL-8 gene promoter. However, the promoter region containing the intact NF-κB binding site without the upstream sequence (-133– -85) did not support the response to LPA (Fig. 4A), suggesting that the
NF-κB binding site is necessary but not sufficient for LPA-induced transcriptional activation. It seems that the full activation of the IL-8 gene promoter relies upon NF-κB and another upstream synergistic factor, likely AP-1. The role of the NF-IL-6 site appears to be negated when the AP-1-binding site is intact, a corollary consistent with the observation that the deletion of the NF-IL-6 in addition to the AP-1 site had some effect on the response to LPA while point mutation of the NF-IL-6 alone did not have significant outcome (Fig. 4B). An alternative possibility is that the -98–-85 sequence of the IL-8 gene promoter may contain an undefined participating element rather than the NF-IL-6 site.

**LPA induces NF-κB and AP-1 transcriptional activity in ovarian cancer cells**

LPA has been shown to induce NF-κB activity in murine fibroblasts and neuronal cells (41,43). We next determined whether LPA indeed stimulates NF-κB and AP-1 transcriptional activity in ovarian cancer cells. Ovarian cancer cell lines were transfected with pNF-κB-Luc or p3xTRE-Luc luciferase constructs. In pNF-κB-Luc, the luciferase reporter gene is driven by a basic promoter element (TATA box) joined to 5 tandem repeats of NF-κB binding sites. The p3xTRE-Luc was constructed as we described previously in which 3 consensuses AP-1 binding sites are placed upstream of the minimal thymidine kinase (TK) promoter to control transcription of the luciferase gene (31). LPA strongly induced luciferase activity in OVCAR-3 and SKOV-3 cells transfected with either pNF-κB-Luc or p3xTRE-Luc (Fig. 5A), indicating that LPA stimulates both NF-κB and AP-1 transcriptional activity in ovarian cancer cells.

**Co-expression of RelA/p65 and c-Jun strongly stimulates transcriptional activity of the IL-8 promoter**

To confirm the importance of the NF-κB and AP-1 transcription factors in the modulation of IL-8 production, we examined whether activation of NF-κB and AP-1 is sufficient to induce
significant transcription from the IL-8 gene promoter in ovarian cancer cells. OVCAR-3 and SKOV-3 cells were cotransfected with pIL8-Luc along with RelA/p65, c-Jun or RelA/p65 plus c-Jun. Overexpression of RelA/p65 and c-Jun is an effective and specific means to upregulate cellular activity of NF-κB and AP-1, respectively (33,44). As shown in Fig. 5B, an increase in NF-κB (via transfection of RelA/p65) or AP-1 (via transfection of c-Jun) induced only minor increases (2 fold) in luciferase activity driven from the IL-8 gene promoter. However, coexpression of RelA/p65 and c-Jun in OVCAR-3 or SKOV-3 cells consistently led to 10-20 fold enhancement in the transcriptional activity of the IL-8 promoter (Fig. 5B), confirming a synergistic effect of NF-κB and AP-1 on IL-8 gene expression.

**LPA induces IL-6 and IL-8 production through Edg LPA receptors**

To determine if the effect of LPA on IL-6 and IL-8 production is mediated by LPA receptors, we examined a number of cell types in search for non-responsive cell lines. As described above, the breast cancer cell line SKBr-3 had minimal IL-6 and IL-8 production in response to LPA (Fig. 1). Northern blot analysis of poly A+ RNA extracted from cancer cell lines confirmed mRNA expression of one or more of Edg LPA receptors in the ovarian cancer cell lines OVCAR-3, SKOV-3, Dov-13, 2780R, HEY, OVCA432, and Caov-3 as well as in the LPA-responsive breast cancer cell lines BT549 and MDA468 (Fig. 6). However, only trace levels of LPA$_2$ and LPA$_3$ transcripts but not that of LPA$_1$ were detectable in SKBr-3 cells compared to the other LPA-responsive cell lines (Fig. 6), an observation suggesting that lack of LPA-induced IL-6 and IL-8 production in SKBr-3 cells could be due to insufficient expression of functional LPA receptors.

To determine whether enforced expression of LPA receptors could restore IL-6 and IL-8 production in response to LPA, SKBr-3 cells were transiently transfected with each of the Edg LPA receptors, LPA$_1$, LPA$_2$ and LPA$_3$ which were tagged with the Flag M2 at the N-terminal. As
demonstrated in Fig. 7A, transfection of each LPA receptor significantly restored LPA-induced IL-6 and IL-8 production in SKBr-3 cells. The maximum response was consistently observed in cells transfected with LPA2. The expression level of LPA2 was actually lower than that of LPA1 or LPA3 as revealed by immunoblotting analysis and immunofluorescent staining of transfected cells (Fig. 7B & 7C), implicating LPA2 as a major receptor mediating IL-6 and IL-8 production.

In parental SKBr-3 or empty vector-transfected SKBr-3 cells, LPA treatment resulted in an apparent decrease in Erk phosphorylation/activation (Fig. 7D), an observation not seen in other mammalian cell lines we tested. To determine whether transfected LPA receptors were able to alter the response to LPA, SKBr-3 cells were co-transfected with each LPA receptor and a GFP vector. GFP-positive cells were isolated by FACS and analyzed for Erk phosphorylation in response to LPA. As shown in Fig. 7D, LPA induced significant Erk phosphorylation in a dose-dependent manner in cells transfected with each of the three LPA receptors, indicating that the transfected LPA receptors were indeed functional in the recipient cells.

Overexpression of LPA receptors in ovarian cancer cells enhances responsiveness to LPA

Most ovarian cancer cell lines express one or more isotypes of LPA receptors and produce IL-6 and IL-8 upon stimulation with LPA. To confirm the involvement of the Edg receptors in LPA-induced IL-6 and IL-8 production in ovarian cancer cells, we asked whether over-expression of LPA receptors sensitizes cells to the effect of LPA. To determine the effect of over-expression of LPA receptors on LPA-induced IL-6 and IL-8 production, we developed an efficient gene transfer system using lentivirus engineered to express LPA receptors in mammalian cells. SKOV-3 cells endogenously express LPA1 and low levels of LPA2 or LPA3 (Fig.6). The cell line responded to LPA with modest levels of IL-6 and IL-8 production compared to Dov-13 cells (Fig. 1). We infected SKOV-3 cells with control virus (pLenti6-LacZ) or with lentivirus expressing each of the
LPA receptors (pLenti6-LPA₁, pLenti6-LPA₂, pLenti6-LPA₃). As presented in Fig. 8, expression of each of the LPA receptor delivered by lentiviruses sensitized LPA-induced IL-6 and IL-8 production when compared to control virus-transduced cells. In particular, the pLenti6-LPA₂ virus-transduced cells showed optimal response to LPA, further supporting the potent capability of this receptor to couple LPA to IL-6 and IL-8 expression. Similar results were obtained when another cell line (OVCAR-3) was analyzed (data not shown).

**LPA-induced IL-6 and IL-8 production is highly sensitive to inhibition of the p38 MAP kinase**

As an initial effort to identify the intracellular signaling pathways involved in the ability of LPA to induce IL-6 and IL-8, we examined the effects of pharmacological inhibitors of signal transduction pathways implicated in LPA signaling. LPA has been shown to activate the PI3K-Akt cascade and multiple MAPK family members including Erk, c-Jun N-terminal kinase (JNK) and the p38 MAP kinase (1,9). We evaluated the PI3K inhibitor wortmannin, the MEK-1 inhibitor PD98059, the JNK inhibitor SP600125 and the p38 MAP kinase inhibitor SB203580 (45). The concentrations of the inhibitors assessed prevented LPA-induced phosphorylation of their target downstream kinases by 60 to >90% in OVCAR-3 cells (data not shown). As presented in Table 1, inhibition of PI3K with wortmannin only modestly reduced LPA-triggered IL-6 and IL-8 production in OVCAR-3 cells (<20%), suggesting a non-specific interference or a modest contribution of the PI3K pathway in LPA-mediated IL-6 and IL-8 production. The MEK-1 inhibitor PD 98059 had slightly greater but still modest effects (<35%, Table 1). The ability of LPA to induce IL-6 and IL-8 generation was most sensitive to inhibition of JNK and the p38 MAP kinase. In particular, the p38 inhibitor SB203580 at 5 μM decreased the activity of LPA by nearly 90% (Table 1). These results suggest that the p38 MAP kinase and probably also JNK are important intracellular mediators of LPA-induced IL-6 and IL-8 production in ovarian cancer cells.
DISCUSSION

As a prototypic ligand of G protein-coupled receptors, LPA has been a subject of extensive research in the field of signal transduction and physiology (1). Its potential role in the pathogenesis of human cancer is a relatively new concept. Although the earliest work that implicated LPA in human cancer development utilized ovarian cancer as a model (2-4, 6), the relevance of LPA to other types of human malignancies has been suggested by several recent studies. For instance, the LPA2 receptor is overexpressed in differentiated thyroid cancer (46). In prostate cancer cells, LPA acts as an autocrine growth factor and one or more of LPA receptors are expressed in prostate cancer cell lines (47, 48). Additionally, autotaxin/Lyso-PLD, a key enzyme in LPA production from lysophosphatidylcholine (LPC) (40), is overexpressed in various human malignancies including renal carcinomas, breast cancers and brain tumors (49,50), implying involvement of LPA production and signal amplification in a variety of human cancers. Recent studies demonstrated that LPA stimulates expression of a number of genes which are involved in the promotion of angiogenesis and metastasis, such as VEGF (36), IL-8 (12) and uPA (37), highlighting the possibility that LPA may contribute to cancer development and progression through regulation of gene expression.

As both IL-6 and IL-8 are strongly implicated in ovarian cancer progression and prognosis (13-15, 17-20), it is critically important to understand the mechanisms underlying overexpression of these factors in ovarian cancer and to explore strategies to restrain their expression. When ovarian cancer cells are analyzed for transcriptional changes in response to LPA, the IL-8 gene is dramatically induced by LPA (12), suggesting that it represents a target gene of LPA. The effect of LPA on IL-6 production has not been described previously. In the current study, we demonstrated that IL-6 is also a major target gene of LPA in ovarian cancer cells. Our results indicate that the effect of LPA on IL-6 and IL-8
production is mediated by specific LPA receptors. Overexpression of LPA receptors, LPA₁, LPA₂ or LPA₃, reconstituted IL-6 and IL-8 generation in an otherwise non-responsive breast cancer cell line and enhanced the sensitivity to LPA in responsive ovarian cancer cell lines. Although all the three LPA receptors are capable of linking LPA to IL-6 and IL-8 generation as suggested by transfection/overexpression experiments, our results have consistently showed that the LPA₂ receptor is more effective than the LPA₁ and LPA₃ receptors in this particular response to LPA. The results suggest that when expressed at endogenous levels, LPA₂ may act as a primary receptor that links LPA to IL-6 and IL-8 gene expression. Although our Northern blot analysis showed the presence of LPA₂ mRNA in some but not all ovarian cancer cell lines examined, several recent studies demonstrated widespread expression of LPA₂ in primary ovarian cancer cells and in almost all ovarian cancer cell lines as analyzed by RT-PCR (51,52).

Our RNA stability analysis and luciferase assays indicate that LPA induces transcriptional activation of the IL-8 gene with little contribution from increased stability of RNA. Detailed characterization of the IL-8 gene promoter revealed that transcription factors, namely NF-κB and AP-1, are likely to mediate the effect of LPA. A synergism between NF-κB and AP-1 seems to be critical for LPA–induced transcriptional activation of the IL-8 gene. Our results also suggest that the p38 MAP kinase and JNK are important intracellular mediators of LPA-induced IL-6 and IL-8 production in ovarian cancer cells.

IL-8 may exert its detrimental effect in ovarian cancer through promoting neovascularization. IL-8 induces chemotaxis in neutrophils and endothelial cells and is angiogenic in vivo as shown in the corneal model of angiogenesis (53). Angiogenesis is potentially a rate-limiting step for the growth and spread of most human malignancies including ovarian cancer (17, 18, 54, 55). In nude mouse xenograft models, the ability of ovarian cancer cell lines to produce IL-8 and VEGF is associated with increased tumorigenicity, angiogenesis and ascites formation (17, 18). Compared to IL-8, a role for IL-6 in
angiogenesis is controversial. Although IL-6 may inhibit endothelial cell proliferation (56), it increases the motility and proper alignment of endothelial cells (57). In the ovary, strong evidence suggests that IL-6 is produced and participates in vascular remodeling during the development of ovarian follicles (58). Several recent studies suggest a role of overexpression of IL-6 and IL-8 (19, 20), particularly IL-6 (59), in the development of a drug resistant phenotype in cancer cells. This effect of IL-6 may contribute to the poor prognosis in ovarian cancer patients overexpressing IL-6 (13). It is not clear how the cytokines IL-6 and IL-8 may modulate cellular responses to chemotherapeutic agents although ovarian cancer cells are known to express IL-6 and IL-8 receptors (60, 61).

The dramatic stimulatory effect of LPA on IL-6 and IL-8 production suggests a potential mechanism for elevated levels of IL-6 and IL-8 in the plasma and ascites of ovarian cancer patients. Ascites from ovarian cancer patients contains haematopoietic cells, mesothelial cells and large numbers of tumor cells (2, 62). LPA colocalizes with tumor cells at high concentrations (1-80 μM) in the ascites of ovarian cancer patients (3-5). The high levels of IL-6 and IL-8 in ascites may reflect their synthesis and secretion by ovarian tumor cells under persistent impact of LPA. Besides LPA, we demonstrate that serum is a potent stimulus of IL-6 and IL-8 production (Fig. 2). Serum contains LPA and other phospholipid factors in addition to the well-defined peptide growth factors PDGF and IGF-1 (38-40). The inability of various peptide growth factors to induce generation of IL-6 and IL-8 in ovarian cancer cells underscores the importance of LPA and other lipid mediators in the regulation of these cytokines by serum. LPA levels are low in serum and nonmalignant effusions (3-5, 40, 63). However, when ovarian cancer cells were incubated in serum-containing medium, LPA may be constantly generated from LPC via action of autotaxin/Lyso-PLD as described recently (40), leading to persistent functional concentrations of LPA in the supernatants.

The present study not only elucidated the molecular processes underlying LPA-mediated IL-6 and IL-8 production, but also hinted at potential approaches to control expression of these cytokines in
ovarian cancer. The potent capability of LPA to stimulate IL-6 and IL-8 production in ovarian cancer cells makes it very likely that LPA represents a major regulator of IL-6 and IL-8 expression *in vivo*. Inhibition of LPA or LPA receptors may thus offer a potential strategy to block IL-6 and IL-8 production and thus to control neovascularization and drug resistance in ovarian cancer. This could be achieved through altering LPA production/metabolism or through interfering with LPA binding to its cognate receptors. Increasing LPA metabolism by overexpression of the membrane-bound lipid phosphate phosphohydrolase (LPP) (64-66) has been shown to decrease growth and tumorigenicity of ovarian cancer cells (67). Our results in this report indicate that the LPA$_2$ receptor is the most effective receptor linking LPA to IL-6 and IL-8 production. Interestingly, LPA$_2$ is overexpressed in many ovarian cancer cell lines and in significant portions of primary ovarian cancers (9, 51,52,68). Targeting the LPA$_2$ receptor may constitute an effective method to restrain IL-6 and IL-8 production in ovarian cancer. As homologous deletion of the LPA$_2$ receptor results in minimal phenotypic changes in knockout mice (69), inhibition of the LPA$_2$ receptor may not prove deleterious to normal physiology. Although there are no pharmaceutical inhibitors of the LPA$_2$ receptor available at present, much effort is being expended to identify LPA receptor subtype-specific antagonists (70-72). Indeed, relatively selective inhibitors of LPA$_1$ and LPA$_3$ have already been identified (71, 72). LPA$_2$-selective agonists, such as 14:0 LPA (73), can be used to address this issue from a different perspective. As we showed in Fig. 2, 14:0 LPA stimulated prominent IL-6 and IL-8 production in OVCAR-3 cells. It will be interesting to determine the *in vivo* effects of selective LPA receptor agonists and antagonists on the production of pro-angiogenic factors, angiogenesis and tumorigenicity of ovarian cancer cells.
ACKNOWLEDGEMENTS

The authors are grateful to Dr. IJ Fidler (MD Anderson Cancer Center) for providing the IL-8 promoter-luciferase constructs; Dr. WH Moolenaar (The Netherlands Cancer Institute) for the Flag-LPA1 expression vector, Dr. P Chiao (MD Anderson Cancer Center) for Rel/p65 cDNA and Dr. V Haridas (MD Anderson Cancer Center) for technical assistance with analysis of NF-κB activation.
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FOOTNOTES

The work was supported by the Lynne Cohen Foundation Ovarian Cancer Research grant 80095031 to X.F., by the NIH grants CA82716 and CA64602 to G.B.M., and by the NIH core grant 5P30 CA016672 to MD Anderson Cancer Center.

Abbreviations used: LPA, lysophosphatidic acid; Edg, endothelial cell differentiation gene; IL-6, interleukin-6; IL-8, interleukin-8; AP-1, activator protein 1; NF-κB, nuclear factor kappa B; NF-IL-6, nuclear factor interleukin-6; PA, phosphatidic acid; FBS, fetal bovine serum
**FIGURE LEGENDS**

**Fig. 1.** LPA stimulation of IL-6 and IL-8 production in ovarian cancer cell lines. After starvation in serum-free medium for approximately 24 hours, OVCAR-3, SKOV-3, Dov-13 and SKBr-3 cells (in 6-well plates) were incubated with increasing concentrations of LPA (18:1) for 14-16 hours. Culture supernatants were collected and assayed for IL-6 and IL-8 levels by ELISA analysis. Curves are plotted as amounts of IL-6 or IL-8 (pg) produced by 5X10^5 cells treated with indicated concentrations (μM) of LPA.

**Fig. 2.** Comparison of the effects of LPA, FBS and prototype peptide growth factors. A. OVCAR-3 cells were cultured and prepared as described in Fig. 1. Cells were then incubated for 14 hours with vehicle (control), 18:1, 16:0 or 14:0 LPA (10 μM), 16:0 PA (10 μM), FBS (5%), IGF-1 (50 ng/ml) or EGF (25 ng/ml). IL-6 and IL-8 levels in the culture supernatants of treated cells were quantified by ELISA. B. OVCAR-3 cells were incubated for 5 min with 18:1 LPA (10 μM), EGF (25 ng/ml), IGF-1 (50 ng/ml), PDGF (50 ng/ml) or FBS (5%). Cells were lysed in SDS sample buffer and analyzed by immunoblotting with Akt and Erk phosphorylation-specific antibodies. Reprobing with anti-β-actin antibody was included to show similar levels of loading among samples.

**Fig. 3.** Transcriptional activation of the IL-8 gene by LPA. A. Analysis of IL-8 mRNA stability in LPA-treated and untreated cells. After starvation in serum-free medium, Dov-13 cells were incubated with vehicle (control cells) or 10 μM of 18:1 LPA (LPA-treated cells) for 4 hours before addition of actinomycin D (5 μg/ml) to block RNA synthesis. Total cellular RNA was extracted at 0, 2, 4, 6 and 8 h post-treatment with actinomycin D. RNA samples (25 μg for control cells and 15 μg for LPA-treated cells) were analyzed by Northern blotting with 32P-labeled IL-8 cDNA as probes. Ethidium bromide
staining of 28S and 18S ribosomal RNA on electrophoresis gels was included to show equal loading among the same set of RNA samples. IL-8 mRNA levels were plotted upon quantitative analysis of intensities of IL-8 mRNA hybridization bands by densitometry. The values at 0 h were defined as 100% with other time points presented as relative percentages. **B.** Luciferase analysis of the IL-8 gene promoter. The luciferase reporter construct pIL-8-Luc containing the full-length IL-8 promoter region (−1481– +44) was transfected into Dov-13, SKOV-3 and OVCAR-3 cells. Transfected cells were stimulated for 6 h without (control) or with 10 μM of 18:1 LPA. Cell extracts were assayed for luciferase activity. LPA-induced luciferase activity in each cell line is presented as fold increases with activity in unstimulated, control cells defined as 1. Control experiments in OVCAR-3 cells transfected with pGL2-Luc containing the constitutive SV40 early gene promoter was included to demonstrate the specificity of LPA’s effect on the IL-8 gene promoter. The data are mean ±S.D. of triplicate assays. Similar results were obtained from at least three independent experiments in each cell line.

**Fig. 4.** Deletion and mutation analysis of the IL-8 gene promoter. **A.** Deletion analysis: OVCAR-3 cells were transfected with luciferase reporter constructs containing different lengths of the IL-8 promoter as indicated. Three well-defined transcription factor-binding sites (AP-1, NF-IL-6 and NF-κB) are present along the IL-8 gene promoter as marked. Fold increases in luciferase activity induced by LPA in transfected cells were determined as in Fig. 4B. The data are mean ±S.D. of triplicate assays. In the right column, LPA-mediated increases in luciferase activity were converted to percent activity with that in cells transfected with the construct containing the full-length IL-8 promoter (−1481– +44) defined as 100%. **B.** Mutational analysis: OVCAR-3 cells were transfected with luciferase reporter constructs containing the -133– +44 fragment (-133-Luc) or its mutant forms with each of the three transcription factor-binding sites individually destroyed (AP-1 mutation: AP-1mu-Luc; NF-IL-6 mutation: NF-IL-6mu-Luc; NF-κB mutation: NF-κBmu-Luc). Fold increases in luciferase activity induced by LPA in
transfected cells were determined as in A. Statistically significant decreases in luciferase activity of the mutant constructs compared to that of the wild type vector (-133-Luc), as determined by the Student’s *t* test, are indicated with ** (*p*<0.01) or * (*p*<0.05). The data are mean ±S.D. of triplicate assays, representative of three independent experiments.

**Fig. 5.** LPA activation of NF-κB and AP-1, two synergistic factors involved in the regulation of IL-8 gene expression. **A.** LPA activation of NF-κB and AP-1 in ovarian cancer cell lines. OVCAR-3 or SKOV-3 cells were transfected with the NF-κB-responsive luciferase reporter construct pNF-κB-Luc (*left*) or with AP-1-responsive luciferase reporter construct p3xTRE-Luc (*right*). After starvation, transfected cells were stimulated for 6 h with vehicle (control) or with 10 μM LPA. Cell extracts were prepared and assayed for luciferase activity. LPA-induced luciferase activity was presented as fold increases with activity in unstimulated, control cells defined as 1. The bars are mean ±S.D. of triplicate assays. **B.** Activation of the IL-8 gene promoter by positive regulators of AP-1 (c-Jun) and NF-κB (RelA/p65). OVCAR-3 or SKOV-3 cells were co-transfected with pIL8-Luc along with pcDNA3-c-Jun, pcDNA3-RelA/p65, pcDNA3-c-Jun plus pcDNA3-RelA/p65, or empty pcDNA3 vector. In all combinations, the molar ratio between c-Jun or RelA/p65 expression vector and the reporter pIL8-Luc was adjusted to 4:1 with a fixed total amount DNA used for each transfection. Approximately 72 hours after transfection, cell extracts were prepared and assayed for luciferase activity. The activity in control cells co-transfected with empty pcDNA3 vector was defined as 1. In both A and B, results were normalized on β-galactosidase activity in cells cotransfected with pCMVβ-gal. Similar results were obtained from three independent experiments in each cell line.

**Fig. 6.** Northern blot analysis of mRNA expression of LPA receptors in ovarian and breast cancer cell lines. PolyA⁺ RNA (4 μg) was extracted from cell lines as described in Experimental Procedures and
analyzed by Northern blotting with $^{32}$P-labelled human LPA$_1$, LPA$_2$ and LPA$_3$ cDNAs as probes. Reprobing with $^{32}$P-labeled GAPDH cDNA probe shows equal loading among RNA samples. Northern assays were repeated twice with similar results.

**Fig. 7.** Reconstitution of LPA-induced IL-6 and IL-8 production in non-responsive SKBr-3 cells by transfection with LPA receptors. **A.** SKBr-3 cells were transfected with an empty vector or with an LPA$_1$, LPA$_2$ or LPA$_3$ expression vector. Transfected cells were starved and then stimulated with 10 μM LPA for 24 h. IL-6 and IL-8 production in empty vector- and LPA receptor-transfected cells was determined by ELISA analysis of culture supernatants. Net increases in IL-6 or IL-8 production induced by LPA were calculated by subtracting background IL-6 and IL-8 generated in vehicle-treated cells from levels produced by LPA-treated cells. Results are means ± S. D. of triplicate assays representative of three independent experiments. **B.** Expression of Flag-tagged LPA$_1$, LPA$_2$ and LPA$_3$ receptors in transfected SKBr-3 cells was confirmed by Western blotting with anti-Flag M2 antibody. **C.** The presence of Flag-tagged LPA receptors in transfected SKBr-3 cells was further confirmed by immunofluorescent staining with anti-Flag M2 antibody. The percentages of Flag M2-positive cells in LPA$_1$, LPA$_2$ and LPA$_3$-transfected cells were 18.4 ± 1.6, 10.5 ± 2.1 and 14.9 ± 2.3, respectively, as determined by counting at least 500 cells under a fluorescent microscope. Data are means ± S. D. of triplicates. Similar results were obtained from two independent experiments. **D.** Expression of LPA receptors mediates Erk phosphorylation in response to LPA in SKBr-3 cells. The vector- and LPA receptor-transfected cells were isolated by FACS using GFP fluorescence as described in Experimental Procedures. The cells were starved and stimulated for 5 min with LPA at indicated concentrations. Cell lysates were analyzed by immunoblotting with an Erk phosphorylation-specific antibody followed by re-probing with a phosphorylation-independent Erk2 antibody.
Fig. 8. Sensitization of cells to the effect of LPA in ovarian cancer cells overexpressing LPA receptors. 
A. SKOV-3 cells were infected with pLenti6-LacZ (control), pLenti6-LPA₁, pLenti6-LPA₂ or pLenti6-LPA₃ as detailed in Experimental Procedures. These transduced cells were stimulated for 15 h with various concentrations of LPA as indicated. Production of IL-6 and IL-8 in culture supernatants was determined by ELISA. B. Immunoblotting analysis with anti-V5 antibody confirmed expression of LPA receptors or Lac Z protein in SKOV-3 cells infected with the corresponding lentiviruses.
Fig. 1
Fig. 3
| Length of the IL-8 promoter | Luciferase activity (fold induction by LPA) | % activity |
|----------------------------|--------------------------------------------|------------|
| -1481                      | 7.8±0.23                                   | 100        |
| -546                       | 8.2±0.71                                   | 106        |
| -272                       | 9.7±0.32                                   | 128        |
| -133                       | 8.7±3.4                                    | 113        |
| -98                        | 3.9±0.98                                   | 43         |
| -85                        | 1.6±0.99                                   | 9          |
| -50                        | 1.3±0.17                                   | 4          |

Fig. 4A
Fig. 4B
Fig. 5
| OVCAR-3 | SKOV-3 | Dov.13 | 2780CP | HeY | OVCA432 | Caov-3 | SKBr-3 | BT549 | MDA468 |
|---------|--------|--------|--------|-----|---------|--------|--------|-------|--------|
|         |        |        |        |     |         |        |        |       |        |

- **LPA₁/Edg2**
- **LPA₂/Edg4**
- **LPA₃/Edg7**
- **GAPDH**

**Fig. 6**
Fig. 7A-7C
Fig. 7D
Fig. 8
Table 1. The effects of pharmacological inhibitors of signaling pathways on LPA-dependent IL-6 and IL-8 production in OVCAR-3 cells

|                          | IL-6         |           | IL-8       |           |
|--------------------------|--------------|-----------|------------|-----------|
|                          | quantity (pg) | percent activity | quantity (pg) | percent activity |
| Vehicle                  | 8±2          | -         | 31±9       | -         |
| LPA                      | 186±10       | 100       | 628±98     | 100       |
| LPA+PD98059-12.5 μM      | 147±15       | 79        | 520±43     | 83        |
| LPA+PD98059-25 μM        | 123±10       | 66        | 448±56     | 71        |
| LPA+SP600125-12.5 μM     | 126±17       | 68        | 473±52     | 75        |
| LPA+SP600125-25 μM       | 97±11        | 52        | 332±47     | 53        |
| LPA+SB203580-2.5 μM      | 58±5         | 31        | 159±31     | 25        |
| LPA+SB203580-5 μM        | 25±2         | 13        | 63±16      | 10        |
| LPA+Wortmannin-0.06 μM  | 171±14       | 92        | 547±31     | 87        |
| LPA+Wortmannin-0.12 μM  | 157±12       | 84        | 509±25     | 81        |

OVCAR-3 cells (5x10^5 cells) were treated with 10 μM LPA in the absence or presence of PD98059, SP600125, SB203580 or wortmannin at the indicated concentrations. The inhibitors were added to culture 30-45 min prior to treatment with LPA. IL-6 and IL-8 levels in culture supernatants were measured as described in Fig. 1. The percent activity of IL-6 and IL-8 production is relative to the measurement in cells treated with LPA alone which is defined as 100%.