Up-regulation of interleukin-6 in human ovarian cancer cell via a Gi/PI3K-Akt/NF-κB pathway by lysophosphatidic acid, an ovarian cancer-activating factor

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Bioactive lysophospholipid, lysophosphatidic acid (LPA), is consistently raised in the ascites of patients with ovarian cancer. Interleukin-6 (IL-6) is a pleiotropic cytokine, which is assumed to be involved in ovarian carcinogenesis. However, the regulation of IL-6 in ovarian cancer remains largely unknown. To elucidate the pathogenesis of ovarian cancer, this study investigated how LPA affects IL-6 production in ovarian cancer cells. Experimental results indicated that LPA stimulates IL-6 expression in all ovarian cancer cell lines tested, but not in normal ovarian surface epithelial (NOSE) cells, owing to the lack of LPA-specific Edg4 and/or Edg7 receptors in NOSE cells. This work demonstrated that LPA transcriptionally activates IL-6 expression, which can be totally blocked by the pertussis toxin, indicating that Gi-mediated signaling is critically involved in inducing IL-6 by LPA. Pharmacological and genetic inhibition assays revealed that Gi-mediated PI3K activation phosphorylated downstream Akt and subsequently induced NF-κB activation causes the induction of IL-6 by LPA in SK-OV-3 cells. In summary, data presented here demonstrate that LPA is an important inducer of IL-6 and LPA-regulated IL-6 expression via a Gi/PI3K-Akt/NF-κB pathway in ovarian cancer cells, providing molecular therapeutic targets for treating ovarian cancer.

Introduction

Epithelial ovarian cancer is characterized by the production of large volumes of ascites and rapid growth of solid intraperitoneal (i.p.) tumors. Malignant ascites from ovarian cancer patients have been found to contain ovarian cancer-activating factors that promote tumor growth both in vitro and in vivo (1,2). Among these, lysophosphatidic acid (LPA) accounts for most of the ability of ascites to activate ovarian cancer cells (3,4). Various cells, including macrophage, mesothelial cells, activated platelets, endothelial cells and ovarian cancer cells themselves, contribute to the raised concentrations of LPA in ascites (4-6). LPA is a naturally occurring phospholipid that exhibits pleiotrophic biological activities via the interaction with specific G protein-coupled receptors, LPA1/Edg-2, LPA2/Edg-4 and LPA3/Edg-7 (7). Various lines of evidence suggest that LPA signaling is involved in the initiation, progression and metastasis of ovarian cancer. LPA may increase ovarian cancer cell proliferation by directly increasing the level of cyclin D1 (8), and protecting ovarian cancer cells from apoptotic cell death induced by platinum, leading to a poorer prognosis associated for ovarian cancer (9). LPA also increases the production of potent growth factors, such as VEGF and IL-8, which facilitate tumor angiogenesis (10,11). Furthermore, LPA is likely to promote matrix metalloproteinase activation and up-regulate urokinase plasminogen activator secretion in ovarian cancer cells, resulting in more aggressive behavior by extracellular stromal breakdown (12,13).

Interleukin-6 (IL-6) is a secreted, multifunctional glycoprotein. IL-6 acts through a hexametric cytokine receptor complex composed of an IL-6-specific receptor alpha chain (gp80) and a signal transducer gp130, and then triggers signaling cascades through the Jak/STAT, Ras/MAPK and PI3K-Akt pathway (14). Various arguments have indicated that IL-6 is important in ovarian carcinogenesis. Moreover, in vitro experiments have shown that epithelial ovarian cancer cells constitutively produce IL-6, thus modulating the host immune response to ovarian cancer (15,16). Increased IL-6R alpha expression and constitutive STAT3 activation also have been found to associate with ovarian cancer proliferation (47). In ovarian cancer patients, the IL-6 level in serum and ascites was markedly elevated and appears to be a significant prognostic factor (17,18). Additionally, elevated concentrations of IL-6 correlated with a poor initial response to paclitaxel chemotherapy, and ovarian cancer cells expressing IL-6 may prevent cisplatin-induced apoptosis (19,20). Furthermore, IL-6 displayed increased chemotactic and/or chemokinetic activity and overall invasiveness for ovarian cancer cells (21). Despite IL-6 being critically involved in ovarian carcinogenesis, the mechanism for the regulation of IL-6 in ovarian cancer remains largely mysterious.

Recently, LPA has been found to induce IL-6 expression in ovarian cancer cells (22), and a new LPA3 receptor-selective agonist, metabolically stabilized LPA analog (2S)-OMPT, was shown to substantially induce IL-6 (23). This study demonstrated that LPA enhanced IL-6 expression in ovarian cancer cells but not in normal ovarian surface epithelial (NOSE) cells. The selective induction of IL-6 by LPA was attributed to the expression of Edg4/Edg7 receptors in ovarian cancer cells. Furthermore, Gi-mediated PI3K-Akt/NF-κB activation is the major signaling pathway responsible for IL-6 induction by LPA. The experimental results presented here clarify the role of LPA in the oncogenesis of ovarian cancer.
Materials and methods

Cell culture

The ovarian cancer cell lines OVCAR-3, Caov-3 and SK-OV-3 were obtained from the American Type Culture Collection (Manassas, VA). OVCAR-3 was cultured in RPMI-1640 medium with 20% fetal bovine serum (Life Technologies, Paisley, UK) and 10 μg/ml gentamicin (Sigma, St Louis, MO). Moreover, SK-OV-3 and Caov-3 were cultured in Dulbecco’s modified Eagle medium with 10% FCS. NOSE cells were harvested as described previously (24). Primary NOSE cells were cultured in a 1:1 mixture of M199/ MCDB105 medium (Sigma, St Louis, MO) supplemented with 15% fetal bovine serum (Life Technologies) and penicillin-streptomycin (100 IU/ml-100 μg/ml; Life Technologies). Moreover, human umbilical vein endothelial cells (HUVECs) were cultured in M199 medium supplemented with 20% fetal bovine serum, endothelial cell growth supplement (Intracel, Rockville, MD), heparin, 1-glutamine, penicillin and streptomycin, in a humidified atmosphere of 95% air and 5% CO2 at 37°C. To prevent the various individual specimens from causing genetic variation in the sample, HUVECs from five or more different donors were pooled together. The following experiments were performed using HUVECs at no more than five passages.

Antibodies and reagents

1-Oleoyl-1P, pertussis toxin (PTX), LYS24002, Wortamadin, PD98059 and PDTC were purchased from Sigma (St Louis, MO). LPA was dissolved in (vehicle) PBS containing 1% fatty acid-free bovine serum albumin (Sigma, St Louis, MO). Recombinant human IL-6 and human IL-6-neutralizing antibody were obtained from R&D systems (Minneapolis, MN). Antibody to human phospho-Erk, Erk, phospho-p38, p38 and p65 were from Santa Cruz Biotechnology. Antibodies to Akt and phospho-Akt were purchased from Upstate Biotechnology. Finally, the dominant-negative (dn) mutant of the Akt vector was kindly provided by Dr R.H.Chen, of the Institute of Molecular Medicine, National Taiwan University, Taipei, Taiwan.

Enzyme immunoassay

The IL-6 levels of the cell culture supernatant were determined using EIA with commercially available kits (R&D Systems). Each measurement was performed in triplicate, and the average value then was recorded as pg/ml.

Plasmid construction

The hIL-6 promoter in a luciferase activity reporter system was constructed and modified as described elsewhere (25). Briefly, the 1.2-kb hIL-6 promoter region was amplified from SK-OV-3 total cellular DNA using primers F (5'-GGAAGATCTTCTCTCGAAGAGACATTCTGTA-3') and R (5'-CGAAGCTTGGGCCAGAATGAGCCCTGAGGATC-3'); the underlined nucleotides represent the βII and HindIII sites, respectively. Moreover, the PCR fragment was cloned into a pGL2-basic vector (Clontech, Palo Alto, CA) to produce pHIL6-1.2K.

Reporter assays

Transfections of pHIL6-1.2K into SK-OV-3 cells were performed in six-well plates using the TransFast Transfection Reagent (Promega) method. At 24 h after transfection, cells were serum-starved for 24 h, and then pre-treated with various reagents. To control the transfection efficiency, cells were co-transfected with pSV-β-galactosidase, and data normalizations after all transfections were conducted using triplicate cultures.

RT-PCR

Total RNA was isolated from cultured cell lines using RNAzol B reagent (Biotech Laboratories) according to the instructions of the manufacturer and then cDNA was prepared from 2 μg of total RNA with random hexamer primers according to the cDNA synthesis ImProm-II protocol (Promega). The specific oligonucleotide primer pairs for certain genes and the expected sizes of the PCR products were as follows: Edg-2, 5'-CACAATAGGGGCTTACGAGCCCA and 5'-TCCCATTTGAGATGTCGTTGTC, 621 bp; Edg-4, 5'-GGGCGGCTCTACACTGGCTCTGAGGCTCTG and 5'-CCGGCGGCTCTACACTGGCTCTGAGGCTCTG; Edg-7, 5'-CTGAAGTTTAAAACAGGCCC and 5'-GAGGGTTTGTTTCTCCTGGA, 402 bp; and beta-actin, 5'-CTCTTACATAGGACCTTGAG and 5'-TCATGAGGTACTGAGGACCTG, 305 bp. IL-6, 5'-CTTGGTGCTTCTGGTCTCTG and 5'-AGGAAGCTTCTTTAAGGCGC, 609 bp.

Synthesis of NF-κB decoy oligonucleotides and treatment

This study used synthetic double-stranded oligonucleotides (ODNs) as ‘decoy’ cis elements to block the binding of nuclear factors to promoter regions of targeted genes, thus inhibiting gene transactivation. The following sequences of the phosphorothioate ODN were utilized: NF-κB decoy ODN: 5'-CCTTGAAGGTTCCCTCC-3' and 3'-GGAACCTCCC-TAAGGGAGG-5'; AP-1 decoy ODN: 5'-TGTCGTAGCTGTGTC-3' and 3'-CAGACTTGGTACA-3'; scrambled decoy ODN: 5'-TTGCGCATTCT-GACCTAGC-3' and 3'-AAGGGCATGGCTAAGG-5'. The ODN was mixed with an equal volume (10:1) of TransFast for 15 min and then incubated with the cells in serum-free medium.

Western blotting

Cells were lysed in a lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 1 mM NaF, 1 mM Na3VO4, 2 mM phenylmethysulfonyl fluoride, 1 μg/ml aprotinin and leupepin in PBS). The lysates were centrifuged at 12,000 r.p.m. for 25 min at 4°C. The protein concentration then was measured using a Bio-Rad protein assay (Hercules, CA). A 50-μg protein sample was separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membrane, and immunoblotted with various antibodies. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies, followed by an enhanced chemiluminescent detection system (ECL, Boehringer Mannheim).

Electrophoretic mobility shift assay

Nuclear extracts were prepared using SK-OV-3 cells in extraction buffer (420 mM KCl, 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.2 mM EDTA and 20% glycerol) containing protease inhibitors (0.5 mM dithiothreitol, 0.5 mM phenylmethysulfonyl fluoride and 10 μg/ml leupepin). The nuclear extracts were incubated in binding buffer with double-stranded oligonucleotides for the classical consensus binding sites of NF-κB (5'-AGTTGAGGGGCTTCCAGGCCG-3'), and the binding sites of Oct1 (5'-TGTCGAGCTGAATCACCATAGGAA-3') were labeled using DIG-d11-dUTP by a DIG gel shift kit (Roche Diagnostics, Indianapolis, IN). Samples were separated on non-denaturing 4% polyacrylamide gels in 0.5× TBE and then transferred onto nitrocellulose membranes. DIG-labeled probe-protein complexes were detected using horseradish peroxidase-conjugated anti-DIG antibodies, and visualized using an ECL system (Boehringer Mannheim).

Statistics

In this study, each experiment was performed in triplicate and all experiments were repeated at least three times on different occasions. A paired Student’s t-test was used to evaluate statistically significant differences in IL-6 protein levels between the LPA treatment groups and the vehicle control group. P < 0.05 was selected as the statistically significant value.

Results

LPA enhances IL-6 expression in human ovarian cancer cells, but not in NOSE cells

Whether LPA directly regulates IL-6 expression in ovarian cancer cells was determined using EIA. SK-OV-3 cells were serum-starved for 24 h, then treated with vehicle or 20 μM LPA for various periods. The experimental results displayed that upon LPA treatment, IL-6 was rapidly induced at 8 h, and the level of IL-6 proteins steadily increased until 24 h (Figure 1A). A change in the level of IL-6 with varying concentrations of LPA was further investigated. Upon stimulation of 5 μM LPA, the expression of IL-6 was significantly elevated in a dose-dependent manner (Figure 1B). Moreover, the level of IL-6 protein could also be markedly up-regulated by LPA in OVCAR-3 and Caov-3 ovarian cancer cells, a phenomenon we did not see in the control group (untreated) or in the vehicle group (PBS containing 1% fatty acid-free bovine serum albumin). Meanwhile, no enhancement of IL-6 production by LPA was observed in NOSE cells or HUVECs (Figure 2A), indicating a cell type-specific induction of IL-6 by LPA. As LPA induces various cellular responses by interacting with specific cell-surface G protein-coupled receptors of the Edg family, RT-PCR was used to differentiate the expression pattern of Edg family genes in these cells. Figure 2B showed that ovarian cancer cells express Edg2, Edg4 and Edg7 mRNA while NOSE cells and HUVECs only express Edg2 mRNA, suggesting the LPA enhances IL-6 production via Edg4 or Edg7 activation.
The Gi/PI3K–Akt pathway is required for LPA-augmented IL-6 expression in SK-OV-3 cells

To investigate the role of LPA on hIL-6 gene transcriptional regulation, we examined whether LPA can activate the hIL-6 promoter in a reporter system. A 1200-bp promoter region upstream of the first hIL-6 exon was cloned into the pGL2-basic luciferase reporter vector to produce phIL6-1.2K. This reporter plasmid was transfected into SK-OV-3 cells and, as shown in Figure 3A, was potently activated (3.2 ± 0.5-fold) by LPA. To confirm that activation of the hIL-6 promoter was mediated via a PTX-sensitive Gi-dependent pathway, transfected SK-OV-3 cells were pre-treated with 100 ng/ml PTX before being LPA stimulated. LPA-induced phIL6-1.2K activity was inhibited by PTX, indicating that LPA transcriptionally up-regulates IL-6 expression through Gi-mediated signaling using a reporter gene assay (Figure 3A). However, the induction of IL-6 mRNA by LPA could be entirely abolished by pre-treatment with 100 ng/ml PTX (data not shown). Together, these results indicate that LPA may selectively enhance IL-6 expression in ovarian cancer cells via an Edg4/Edg7-mediated Gi-dependent pathway. Accordingly, it is interesting to unravel the cytoplasmic signaling involved in the LPA-mediated IL-6 up-regulation in ovarian cancer cells. Previous investigations have demonstrated that the major LPA signaling through the PTX-sensitive path is the Gi-mediated activation of PI3K–Akt and MAP kinase (26). As expected, LPA treatment induced substantial Akt, ERK and p38 phosphorylation in SK-OV-3 cells, which could be inhibited by PTX and selectively blocked by the chemical inhibitors LY294002, SB203580 and PD98059, respectively (Figure 3B). However, only the PI3K inhibitors, LY294002 and Wortmannin, could markedly inhibit LPA-induced IL-6 mRNA expression according to RT–PCR (Figure 3C). Under identical circumstances, the induction of IL-6 mRNA expression in NOSE cells was not significantly affected by these inhibitors.

Fig. 1. LPA induced IL-6 expression time dose dependently in human ovarian cancer cells. (A) LPA induces IL-6 protein expression time dependently. SK-OV-3 cells were serum-starved for 24 h prior to 20 μM LPA or vehicle treatments with various lengths. The IL-6 protein level was detected via EIA. (B) LPA induces IL-6 protein expression dose dependently. SK-OV-3 cells were serum-starved for 24 h prior to 24-h LPA treatment with various concentrations as indicated. The IL-6 protein level was detected via EIA. Data are presented as mean ± SD from three independent experiments. A paired Student’s t-test was used to evaluate statistically significant differences in IL-6 protein levels between the LPA treatment groups and the vehicle treatment group: *P < 0.05.

Fig. 2. LPA enhances IL-6 expression in human ovarian cancer cells, but not in NOSE cells. LPA-specific receptors Edg2, -4 and -7 expression patterns in ovarian cancer cell lines, NOSE and HUVECs. (A) LPA enhanced IL-6 expression in ovarian cancer cell lines, but not in NOSE cells or primary culture of HUVECs. Ovarian cancer cell lines were serum-starved for 24 h, while HUVECs and NOSE cells were serum-starved for 12 h, and cells were then treated with 20 μM LPA for 24 h. The IL-6 protein level was detected via EIA. A paired Student’s t-test was used to evaluate statistically significant differences in IL-6 protein levels between the LPA treatment groups and the vehicle treatment group: *P < 0.05. (B) LPA-specific receptors Edg4 and -7 are mainly expressed in ovarian cancer cell lines, but not in NOSE or HUVECs. Total RNA of the culture cells was extracted under normal culture conditions as described in the Materials and methods. LPA-specific receptors Edg2, -4, -7 and beta-actin were detected using specific primers by RT–PCR.
Fig. 3. LPA may selectively enhance IL-6 expression in ovarian cancer cells via an Edg4/Edg7-mediated Gi-dependent pathway and the Gi/PI3K–Akt pathway is required for LPA-augmented IL-6 expression in SK-OV-3 cells. (A) LPA-induced IL-6 promoter luciferase activity was abolished by the G-protein inhibitor PTX. SK-OV-3 cells were serum-starved for 24 h and then pre-treated with 100 ng/ml of PTX for 1 h prior to 20 μM of LPA treatment. After 6 h, the luciferase activity of the IL-6 promoter was measured by luminometer. To control for transfection efficiency and experimental variations, SK-OV-3 cells were co-transfected with pSV-β-galactosidase, which constitutively expresses the β-galactosidase to normalize the fold of induction. Data are presented as mean ± SD from five independent experiments. A paired Student’s t-test was used to evaluate statistically significant differences in fold of induction between the LPA treatment groups and the PTX treatment group; *P < 0.05. (B) LPA induced p38, Erk and Akt phosphorylation via a PTX-sensitive G protein-mediated signaling. SK-OV-3 cells were serum-starved for 24 h, then pre-treated with different chemical inhibitors for 1 h prior to 20 μM LPA treatment. After 30 min, total protein was collected and the expression level of the indicated protein was measured by western blot with specific antibodies. (C) LPA induced IL-6 mRNA expression mainly through PI3K, but not p38 or Erk. SK-OV-3 cells were serum-starved for 24 h, then pre-treated with SB203580 (5 μM), PD98059 (50 μM), Wortmannin (100 ng/ml), LY294002 (50 μM), PTX (100 ng/ml) or vehicle (0.5% DMSO) for 1 h prior to 20 μM LPA treatment. After 6 h, the total RNA was collected and the expression level of the indicated protein was determined by RT–PCR with specific primers. (D) dnAkt inhibits LPA-induced IL-6 up-regulation. SK-OV-3 cells were transiently transfected with dnAkt or pcDNA3 vector for 24 h. Cells were then serum-starved for 24 h before being treated with 20 μM LPA. Total protein was collected in 30 min and the status of phospho-Akt/Akt was determined by western blotting (upper). The conditioned medium of treated cells was gathered after 24 h and the expression level of IL-6 protein was determined by EIA. Data are presented as mean ± SD from three independent experiments. A paired Student’s t-test was used to evaluate the statistically significant difference in IL-6 induction by LPA between the dnAkt transfected group and the pcDNA3 dn-Akt transfected group; *P < 0.05.
of IL-6 promoter luciferase activities or the IL-6 protein level was selectively blocked by LY294002 and Wortmannin in SK-OV-3 cells (data not shown); in several cancer cell lines, IL-6 mRNA up-regulation is paralleled with those of IL-6 protein (46). Moreover, to confirm the role of the PI3K-Akt pathway in LPA-induced IL-6 expression, a dn Akt vector was transfected into SK-OV-3 cells to determine its effect on LPA-mediated IL-6 expression, a 72-h transient-transfected test was used in this experiment. The transfection efficiency of SK-OV-3 by our transfection protocol is ~23% by calculating the determined expression of green fluorescent protein. The inhibition ratios of phospho-Akt/Akt in parent SK-OV-3 (wild-type, denoted as WT), dn Akt and vector control (pcDNA-3) group are 2.1, 1.5 and 1.9, respectively, by densitometer. The inhibition rate of Akt activation is ~26% (dn Akt versus pcDNA-3 group). As for the LPA regulates the IL-6 expression test, the inhibition rate of dn Akt versus pcDNA-3 group is 27% (dn Akt versus pcDNA-3 group, 212 and 285 pg/ml, respectively, P < 0.05). The transient transfected-dn Akt cell line exhibited a marked reduction in LPA-induced IL-6 expression when compared with the vector control or parent SK-OV-3 (Figure 3D). The above findings indicate that the PI3K and its downstream Akt are activated by LPA. LPA mediates the signaling to up-regulate the IL-6 level.

Critical involvement of NF-κB activation in the induction of IL-6 by LPA

The upstream promoter sequences of IL-6 contain conserved putative binding sites for NF-κB and AP-1 (25). We used synthetic double-stranded ODNs as decoy cis elements to check the role of NF-κB and AP-1 in the induction of IL-6 by LPA. The protein level of LPA-induced IL-6 detected by EIA was significantly inhibited by decoy NF-κB in a dose-dependent manner: treatment with high-dose decoy NF-κB decreases LPA-induced IL-6 expression by nearly 92% despite a high-dose decoy-AP-1 also decreasing LPA-induced IL-6 by nearly 27%. However, we thought that it showed that a non-specific interference or a modest contribution of the decoy-AP-1 in LPA-induced IL-6 might also exist (Figure 4A). Consistent with EIA results, IL-6 mRNA was significantly inhibited by the specific NF-κB chemical inhibitors, PDTC and BAY11-7082, using RT-PCR (Figure 4B).

We then clarified the relationship between the Gi/PI3K-Akt signal transduction pathway and NF-κB activity. The NF-κB activation includes nuclear translocation and DNA-binding activity. The LPA-enhanced NF-κB nuclear translocation (Nuclear p65) was inhibited by PDTC, LY294002 and PTX (Figure 4C). On the other hand, the DNA binding activity of NF-κB increased by LPA was significantly inhibited by LY294002 and PTX (Figure 4D) as well. Super-shift assays were carried out to confirm the presence of p50 and p65 binding to the NF-κB-binding site, showing that the specific protein-DNA-binding activity was super-shifted by the addition of anti-p65 or anti-p50 antibodies (data not shown). These results verified that LPA up-regulates IL-6 expression by the Gi/PI3K-Akt/NF-κB signal transduction pathway in SK-OV-3 cells.

Discussion

LPA is present in a raised concentration in the ascites and plasma obtained from patients with ovarian cancer. Previous reports have shown that the plasma LPA levels in ovarian cancer patients ranges from 1.0 to 43.1 μM (28,29); therefore, the concentration of LPA (20 μM) utilized in this study was physiological and can be achieved in vivo. LPA critically influences ovarian carcinogenesis, with LPA-related metabolites present in the ascites of ovarian cancer patients stimulating both anchorage-dependent and anchorage-independent growth of ovarian cancer cells, preventing apoptotic cell death induced by platinum, and increasing the production of factors involved in neovascularization and metastasis (1,9,30). This study demonstrated that LPA significantly enhanced IL-6 expression in ovarian cancer cells but not NOSE cells, owing to the appearance of Edg4/Edg7 receptors in ovarian cancer cells. High amounts of IL-6 are present in the ascites of patients with ovarian cancer, and may confer resistance to apoptosis and anoikis via an autocrine mechanism. The experimental data presented here indicate that LPA is an essential microenvironmental factor inducing IL-6 expression in ovarian cancer patients, and supports previous works suggesting that Edg4/Edg7 may contribute to the deleterious effect of LPA in ovarian cancer (31,32).

The biological responses of LPA signaling are determined by the spectrum of LPA receptors expressed on the cell surface. Ovarian cancer cells have variable Edg2 mRNA and protein levels, and show no consistent change between normal and transformed ovarian cancer cells. Over-expression of Edg2 in ovarian cancer lines has been reported to induce apoptosis, suggesting that Edg2 may be a negative regulator of ovarian epithelial cell growth and metastasis (33). In contrast, Edg4 and Edg7 mRNA expression appears to be elevated in ovarian cancer cell lines compared with NOSE cells (31,32). Previously, LPA has been shown to regulate VEGF and IL-8 expression by activating Edg4 and/or Edg7-mediated signaling (10,22). In agreement with these studies, the experimental results presented here demonstrated that the generation of IL-6 by LPA is linked to the appearance of Edg4 and/or Edg7, highlighting the importance of Edg4/Edg7 in ovarian carcinogenesis.

The Edgs are G protein-coupled receptors. Edg4/Edg7 receptors couple to Gi and Gq, which in turn feed into multiple effector systems (34–36). This study demonstrated that LPA enhances IL-6 expression in ovarian cancer cells via PTX-sensitive G proteins, indicating that Gi-mediated signaling is critically involved in the induction of IL-6 by LPA. Activation of Gi by LPA may stimulate at least three distinct intracellular signals: the inhibition of adenylyl cyclase; the stimulation of the mitogenic RAS-MAPK cascade; and the PI3K-Akt anti-apoptotic pathway (37–39). The analytical results presented here have confirmed that a PI3K-Akt pathway, but not Erk- or p38-dependent pathways, leads to LPA-induced IL-6 up-regulation in SK-OV-3 ovarian cancer cells. This finding differs from previous reports showing that p38 MAPK and probably also JNK are important intracellular mediators of LPA-induced IL-6 production in ovarian cancer cells (22), implying that the signaling pathways leading to the IL-6 induction may vary according to the cell models or stimuli employed. Aberrant PI3K function is crucial in a wide range of important cellular processes associated with ovarian malignancies (40,41). According to this study, the inhibition of PI3K activity would reduce LPA-mediated IL-6 expression and thus provide an effective approach for treating ovarian cancer patients.
PI3K–Akt has been reported to be a possible mediator of post-transcriptional mRNA stabilization (42,43). However, the IL-6 promoter assay showed that LPA induces IL-6 mainly through transcriptional activation. Characterization of the upstream promoter sequences of IL-6 revealed that NF-κB and AP-1 are likely to mediate the effects of LPA. Pharmacological and genetic inhibition assays verified that NF-κB, but not AP-1, is critically involved in the transcriptional activation of IL-6 by LPA. Supporting evidence from previous studies demonstrated that activation of PI3K leads to phosphorylation and activation of NF-κB, and that NF-κB is targeted by Akt in anti-apoptotic signaling (44,45). The development of ovarian cancer is associated with the acquisition of cellular resistance to anoikis, which is a form of apoptosis induced by detachment from the extracellular matrix (27).

Recently, Fang et al. found that both the Edg4 and the p38 MAP kinase are important intracellular mediators of LPA-induced IL-6 in OVCAR-3 cells (22), and Qian et al. found that a new Edg7 receptor-selective agonist, metabolically

Fig. 4. NF-κB is a prerequisite transcriptional factor activated by LPA and mediated Gi/PI3K signaling to up-regulate IL-6 expression. (A) SK-OV-3 cells were transfected with NF-κB decoy ODN, AP-1 decoy ODN or scrambled ODN under serum starving for 24 h and then treated with 20 μM LPA for another 24 h. IL-6 protein expression in the condition medium of the treated cells was detected by EIA. Data are presented as mean ± SD from three independent experiments. A paired Student’s t-test was used to evaluate statistically significant difference in IL-6 induction by LPA between the LPA treated only group and different decoy ODN combined group: *P < 0.05. (B) SK-OV-3 cells were serum-starved for 24 h and then pre-treated with NF-κB-specific inhibitor PDTC or BAY for 1 h prior to 20 μM LPA treatment. Following 6 h, total RNA was gathered and the expression level of IL-6 and beta-actin mRNA was determined by RT-PCR. (C) SK-OV-3 cells were serum-starved for 24 h and then pre-treated with PDTC (100 nM), LY294002 (50 μg/ml) or PTX (100 ng/ml) for 1 h before 20 μM LPA treatment. Nuclear and cytosolic proteins were gathered after 1 h, and the level of NF-κB p65 subunit was determined by western blotting. (D) SK-OV-3 cells were serum-starved for 24 h and then pre-treated with PDTC (100 nM), LY294002 (50 μg/ml) or PTX (100 ng/ml) for 1 h prior to 20 μM LPA treatment. Nuclear proteins were gathered after 1 h, and the binding activities of transcription factors NF-κB and OCT-1 were detected by non-isotope gel shift assay. OCT-1 is a housekeeping transcription factor for loading control.

Fig. 5. Schematic signaling of LPA-mediated IL-6 up-regulation. LPA regulated IL-6 expression mainly through Edg family G protein-coupled receptors mediated PI3K–Akt or MAPK/p38 transduction signaling, NF-κB activation pathway in ovarian cancer cells.
stabilized LPA activate (2S)-OMPT, was shown to induce MAPK, Akt activation and IL-6 release in human OVCAR3 ovarian cancer cells (23). Based on the above new findings, together with our finding here that Gi/Pi3K-Akt/NF-κB signaling mediates the induction of IL-6 by LPA. We conclude that LPA is one of the important inducers of IL-6 in ovarian cancer cells (Figure 5). The experimental result is valuable for developing novel molecular therapies for ovarian cancer.

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