2009

LYSOPHOSPHATIDIC ACID IS A MEDIATOR OF INTERLEUKIN-6 PRODUCTION IN OVARIAN CANCER CELLS

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LYSOPHOSPHATIDIC ACID IS A MEDIATOR OF INTERLEUKIN-6 PRODUCTION IN OVARIAN CANCER CELLS

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

by

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RICHMOND, VIRGINIA
JULY 2009

Acknowledgements

I am very thankful to my advisor, Dr. Frank Fang for giving me the opportunity to learn under his mentorship. He not only taught me about scientific research, but also taught me how to be more efficient in my daily life. The advice and guidance I have received from Dr. Fang will be essential tools for success in my future.

I also want to thank the Fang lab members, Regina Oyesanya, Denise Wu, Abir Mukherjee, and Pei-Lun Lee for being my family away from home. They gave me the support and guidance I needed throughout the training program.

I would finally like to acknowledge Dr. Charles Chalfant and Dr. Murthy Karnam for serving on my supervisory committee. Their scientific suggestions are important for me to complete my thesis on time. I also extend this gratitude to all the members of the Biochemistry Department, who made my stay at VCU an enjoyable experience.

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List of Abbreviations

AA: Arachidonic acid

Ab: Antibody

AP-1: Activator Protein-1

ATF: Activating transcription factor

ATP: Adenosine triphosphate

ATX: Autotaxin

Bcl10: B-cell CLL lymphoma 10

BSA: Bovine serum albumin

bZIP: Basic leucine zipper

C-terminus: Carboxyl terminus

C/EBP: CCAAT enhancer binding protein

CaMK: Camodulin kinase

cAMP: Cyclic adenosine monophosphate

CARMA3: CARD and MAGUK domain-containing protein 3

cDNA: Complementary deoxyribonucleic acid

DMEM: Dulbecco's modified eagle medium

DN: Dominant negative

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

EDG: Endothelial differentiation gene

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EIA: Enzyme Immunoassay

ELISA: Enzyme-linked immuno sorbent assay

ERK: Extracellular signal-regulated kinsae

FBS: Fetal bovine serum

G: Guanine nucleotide

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFP: Green fluorescent protein

GPCR: G protein coupled receptor

GSK-3: Glycogen synthase kinase-3

HGF: Hepatocyte growth factor

IGF: Insulin-like growth factor

IκB: Inhibitor of kappa B

IKK: Inhibitor of kappa B kinase

IL: Interleukin

Jak: Janus kinase

JNK: c-Jun N-terminal kinase

kDa: Kilo Dalton

LAP: Liver-enriched transcriptional activator protein

LIP: Liver-enriched inhibitory protein

LPA: Lysophosphatidic acid

MALT-1: Mucosa associated lymphoid tissue lymphoma translocation gene 1

MAPK: Mitogen-activated protein kinase

NF-κB: Nuclear factor-kappa light chain enhancer of B cells

N-terminus: Amino terminus

PAF: Platelet-activating factor

PAGE: Polyacrylamide gel electrophoresis

PCR: Polymerase chain reaction

PGE2: Prostaglandin E2

PI3K: Phosphoinositol 3-kinase

PKC: Protein kinase C

PKD: Protein kinase D

PPAR: Peroxisome proliferator-activated receptor

PTX: Pertussis toxin

RLU: Relative light unit

RNA: Ribonucleic acid

Rpm: Revolutions per minute

RTK: Receptor tyrosine kinase

RT-PCR: Reverse transcription polymerase chain reaction

S1P: Sphingosine-1-phosphate

SDS: Sodium dodecyl sulfate

siRNA: Small interfering ribonucleic acid

UTR: Untranslated region

VEGF: Vascular endothelial growth factor

WT: wild type

Y: Tyrosine

ABSTRACT

LYSOPHOSPHATIDIC ACID IS A MEDIATOR OF
INTERLEUKIN-6 PRODUCTION IN OVARIAN CANCER CELLS

by David Tran Dang

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

Virginia Commonwealth University School of Medicine, 2009

Director: Xianjun Fang
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Lysophosphatidic acid (LPA) is a naturally occurring bioactive lysophospholipid that mediates a broad range of cellular processes such as cell proliferation, survival, migration and invasion. LPA also plays a potential role in human oncogenesis as suggested by elevated expression of its receptors and its producing enzymes in malignant tissues. In the current study, we demonstrated that LPA is a potent mediator of interleukin-6 (IL-6) production in ovarian cancer. IL-6 is a pleiotropic cytokine which is thought to be an important mediator of ovarian cancer development and progression. Here, we demonstrated that IL-6 levels are indeed increased in the plasma of ovarian cancer patients as compared to normal women. The IL-6 concentrations in ascites of ovarian cancer

patients are even higher than those present in the plasma samples. These results suggest that increased IL-6 are expressed and secreted by ovarian cancer cells, forming a gradient from the ascites to the blood. Ovarian cancer cells indeed produce IL-6 in culture. However, when these cells are starved in serum-free medium, they cease producing IL-6, suggesting that IL-6 is not constitutively expressed, but rather in response to exogenous factors present in serum. We showed that IL-6 expression is not driven by peptide growth factors such as insulin-like growth factor I or epidermal growth factor. Instead, IL-6 expression is most potently induced by the lysophospholipid growth factor LPA. Treatment of ovarian cancer cells with LPA leads to transcriptional activation of the IL-6 gene promoter through activation of the NF-κB and C/EBP transcription factors. LPA also induces tyrosine phosphorylation and activation of Stat-3, a well known intracellular effector of IL-6. However, blockade of IL-6 with a neutralizing antibody only slightly reduced Stat-3 phosphorylation in response to LPA, suggesting that LPA may induce Stat-3 directly or through secondary mediators other than IL-6. Together, these studies demonstrate the role of LPA in regulation of IL-6 production and the underlying mechanism in ovarian cancer.

INTRODUCTION

1.1 Overview

Cancer is often characterized by uncontrolled cell growth, invasion of adjacent tissues and metastasis to other locations in the body. The causes of cancer include abnormalities in the genetic material of the transformed cells. Such abnormalities may be due to the effects of carcinogens such as radiation, chemicals, or infectious agents. Other abnormalities may be acquired through epigenetic changes such as DNA methylation and deregulation of microRNAs. Genetic abnormalities generally affect two classes of genes, oncogenes and tumor suppressor genes. Oncogenes are activated in cancer cells and give the cell properties such as hyperactive growth, protection against programmed cell death, and the ability to become established in diverse tissue environments. Tumor suppressor genes, on the other hand, are inactivated in cancer cells, which results in the loss of normal functions such as accurate DNA replication, cell cycle control, orientation and adhesion within tissues, and interaction with protective cells of the immune system. Cancer can be treated by surgery, chemotherapy, radiation therapy, immunotherapy, or other approaches, depending upon the location and grade of tumors as well as the stage of the disease.

Ovarian cancer arises from different parts of the ovary. The most common form originates from the outer lining, or epithelium of the ovary. Ovarian cancer is the fifth leading cause of death from cancer in women and the leading cause of death from gynecological cancers. Some of the symptoms of ovarian cancer include abdominal pain,

abdominal mass, bloating, back pain, urinary urgency, constipation, tiredness, pelvic pain, abnormal vaginal bleeding, involuntary weight loss and a build-up of ascitic fluid in the abdominal cavity. There are 4 stages of ovarian cancer. The Stage I ovarian cancer is limited to the ovary, stage II involves pelvic extension or implants, Stage III involves microscopic peritoneal implants outside of the pelvis, and stage IV is characterized by the presence of distant metastases to the liver or outside the peritoneal cavity. The cause of ovarian cancer remains largely unknown. Treatment usually involves surgery followed by chemotherapy and/or radiotherapy.

At the advanced Stage III and IV, ovarian cancer is often characterized by formation of large volumes of ascitic fluid. The ascitic fluid from ovarian cancer patients contains ovarian tumor cells and a broad range of potent growth factors [1, 2]. Among them is lysophosphatidic acid (LPA), a naturally occurring phospholipid. The levels of LPA in the plasma of ovarian cancer patients are significantly higher than those in normal controls [1, 3]. LPA levels are therefore a useful biomarker for ovarian cancer. LPA influences many biological processes of tumor cells, including growth, survival, migration and invasion [3, 4]. LPA exerts these effects through G-protein coupled cell surface receptors that are linked to intracellular signaling pathways and ultimate cellular responses. Therefore, understanding the roles of LPA and its receptors in regulation of cellular functions may lead to identification of therapeutic targets for treatment of ovarian cancer and other types of cancer.

1.2 Metabolism of LPA

LPA could be produced by many different cell types including activated platelets [5], endothelial cells [6], fibroblasts [7], adipocytes [8], prostate cancer cells [9] and ovarian cancer cells [4]. LPA is therefore present in body fluids including plasma, saliva, hair follicles and malignant effusions [10, 11]. Certain enzymes targeting phospholipids of cell membranes are responsible for endogenous generation of LPA. In activated platelets, LPA is synthesized by the sequential actions of phospholipase A1 or A2 on serum or membrane phospholipids such as phosphatidylcholine (PC) followed by hydrolytic actions of lysophospholipase D/Autotaxin (ATX) present in the plasma [10, 12]. ATX is synthesized as a full-length, or pre-pro-enzyme, that is proteolytically cleaved in transit along the classical export pathway and secreted as a catalytically active glycoprotein [13]. ATX has intrinsic lysoPLD activity which hydrolyzes lysophosphatidylcholine (LPC) into LPA. The phosphorylation of monoacylglycerol by acylglycerolkinase (AGK) is another source of LPA production [9]. The exact pathways for the generation of LPA in ascites, saliva, seminal and other body fluids remain to be fully elucidated.

There is still ongoing speculation about intracellular production of LPA. In ovarian and other cancer cells, LPA production can be stimulated by cell activation in response to phorbol esters [14], bombesin [14] and LPA itself [15, 16]. The activation of LPA production involves multiple steps catalyzed by various phospholipases.

Lipid phosphate phosphohydrolases (LPP) are a family of enzymes that catalyze the dephosphorylation of LPA [17-19]. There is evidence that expression of these enzymes

reduces LPA levels and compromises LPA-induced cellular functions [17]. In addition to dephosphorylation, LPA can also be converted to phosphatidic acid (PA) by acylation through the action of LPA acyltransferases (LPAAT) [20].

1.3 LPA Receptors and Signal Transduction

The bioactive properties of LPA include the promotion of cell proliferation and survival, enhancement of cell migration and invasion, and the induction of changes in actin cytoskeleton and focal contact organization [2, 4, 21]. These cellular responses are a result of the binding of LPA to its receptors on the plasma membrane, which then initiate a diverse array of signaling pathways. At least seven LPA receptors (LPA_{1-7}) have been identified. LPA receptors are classified into two groups based on their primary structures. They are either members of the endothelial differentiation gene (Edg) family, or fall into the purinergic receptor family (P_2Y). LPA_1/Edg-2, LPA_2/Edg-4 and LPA_3/Edg-7 belong to the Edg family and share about 50-57% homology in their amino acids [22-25]. LPA_4/P2Y9/GPR23 and LPA_5/P2Y5 of the P_2Y family are distant from the Edg family and share only 20-24% homology with the classical Edg LPA receptors, LPA_{1-3} [21, 26]. LPA has also been identified as a ligand for two additional receptors, GPR87 and P2Y10 of the P2Y family [27, 28]. However, the identities of these receptors as additional bona fide LPA receptors need to be more thoroughly investigated.

LPA receptors are G protein coupled receptors (GPCRs). They elicit their activities by coupling to trimeric G proteins subunits, Gα and Gβγ [21-26]. LPA GPCRs couple to diverse G proteins including Gi, Gq and G12/13 to initiate the activation of parallel yet interactive intracellular signaling cascades culminating in physiological responses. Activation of Gq mediates the activation of phospholipase C (PLC) with subsequent

hydrolysis of phosphatidylinositol biphosphate (PIP2) to inositol trisphosphate (IP3), an activator of intracellular calcium release and diacylglycerol (DAG), that activates protein kinase C (PKC) [7, 29]. Gi mediates the inhibition of adenylate cyclase leading to down regulation of intracellular cAMP. Gi or associated Gβ/γ subunit are also linked to activation of Ras and downstream Ras/mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) [30, 31]. Activation of Ras-MAPK and PI3K are critical to LPA-induced cell proliferation, migration and survival [30, 31]. The effects of LPA on stress fiber formation and the cell cytoskeleton occur through the activation of the G12/13-RhoA pathway [32].

LPA signaling inactivation involves internalization of receptor from the plasma membrane. The mechanism for this, however, is not fully understood. LPA may have a role in intracellular signaling. One intracellular target is the proliferator-activated receptor γ (PPAR-γ), which regulates the transcription of genes involved in glucose and fatty acid metabolism, adipocytes differentiation and inflammation processes [33-37]. Although the general functions of LPA receptors are fairly understood, the specific signal transduction cascades and their contribution to the biological functions of LPA remain to be uncovered.

A change in LPA receptor expression is a common event associated with malignant transformation. The Edg LPA receptors are differentially expressed in various tissues [35, 38]. Although LPA_1 is widely expressed and present in both normal and malignant cells, expression of LPA_2 is more restricted. LPA_3 is barely seen in normal tissues [39]. In ovarian and thyroid cancers, malignant transformation is associated with increased expression of LPA_2 (and LPA_3 in ovarian cancer) [40, 41]. LPA receptors are also over

expressed in many other cancer types including endometrioid, colon, and colorectal cancer [42-44]. Recent evidence also suggests that increased expression of LPA receptors correlates with cancer progression processes such as migration and metastasis [40, 41]. Similarly, the presence of LPA in intraperitoneal effusions of ovarian cancer patients may contribute to the progression of malignant cells [4, 40, 45].

LPA influences various cellular processes through its ability to regulate the expression of diverse genes. Microarray analysis of LPA-induced gene expression in an ovarian cancer cell line showed that LPA stimulated expression of many cancer-related genes as our group reported previously [46] . LPA can therefore modulate functions of malignant cells by inducing expression of cytokines, proteases, cell adhesion molecules, proangiogenic factors and anti-apoptotic genes. Evidence suggests that the effect of LPA on gene expression involves transcriptional activation mediated by transcription factors. Transcription factors directly bind to DNA or attach to bound complexes via non-covalent interactions. Many transcription factors consist of one or more DNA binding domain (DBD) [47]. They often possess a trans-activating domain (TAD) and/or a signal sensing domain (SSD) [48]. Transcription factors positively or negatively modulate the expression of their target genes. Functionally, a transcription factor can either be constitutively active (present in the cell all the time) or conditionally active (requiring cell-specific or external signal for activation). In this dissertation, we have focused on LPA-induced expression of Interleukin 6 as a model to understand activation of transcription factors and gene expression induced by LPA.

Figure 1. The human IL-6 gene promoter. The binding sites for the NF-κB, C/EBP and AP-1 transcription factors are indicated.

1.4 Interleukin-6

Interleukin-6 (IL-6) is a secreted, multifunctional glycoprotein. It is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine [49]. IL-6 is secreted by T-cells and macrophages to stimulate the immune system in response to trauma [49]. It is one of the most important mediators of fever and the acute phase response, or response to inflammation. IL-6 can be secreted by macrophages in response to specific microbial molecules [50]. IL-6 acts through a cytokine receptor complex composed of an IL-6 specific receptor alpha chain (gp80) and a signal transducer gp130. This triggers signaling cascades through the Jak/Stat, Ras/MAPK and PI3K-Akt pathways. Several reports indicate that IL-6 is an important modulator of tumor cell progression. For example, in ovarian cancer patients, the IL-6 level in serum and ascites was reported to be elevated [51]. These elevated concentrations also correlate with poor response to drugs and increased chemotactic activity and overall invasiveness for ovarian cancer cells [51]. Despite the critical role of IL-6 in ovarian cancer, the mechanism for regulation of IL-6 expression in the disease is still a mystery.

Several studies have described the general mechanisms for the activation of common transcription factors including activator protein 1 (AP-1), signal transducers and activators of transcription (Stats), specificity protein 1 (Sp-1), CCAAT/enhancer binding proteins (C/EBPs) and nuclear factor-kappa light chain enhancer of B cells (NF-κB). However, specific information of how cellular context might modulate the activities of these proteins in many human malignancies including ovarian cancer is still lacking. Many LPA-target genes harbor binding sites for a common subset of transcription factors in their promoters, suggesting common mechanisms for their regulation by LPA. The human IL-6 promoter contains many of these regulatory elements such as binding sites for the transcription factors NF-κB (−75/−63), C/EBP (−158/−145 and −87/−76) and AP-1 (−283/−277) [52].

MATERIALS AND METHODS

2.1 Reagents

LPA (14:0, 16:0, 18:1) and phosphatidic acid (PA, dipalmitoyl) 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). Anti-Stat-3 and anti-Stat-3 phospho, and anti-IL-6R antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IL-6 neutralizing antibody was obtained from R&D Systems (Minneapolis, MN). Insulin, TRIzol and cell culture reagents were obtained from Invitrogen Inc. (Carlsbad, CA). Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Insulin-like growth factor (IGF) was obtained from Upstate Biotechnology (Lake Placid, NY). Hepatocyte growth factor (HGF) and the IL-6 ELISA kit Quantikine IL-8 were obtained from R & D systems (Minneapolis, MN). Epidermal growth factor (EGF) and platelet derived growth factor (BB isoform) were from Sigma (City and State).

2.2 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 II to IV. 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. There were 30 normal patient samples, and 82 ovarian cancer patient samples. Matched specimens (plasma and ascites) were available from 21 out of 82 ovarian cancer patients.

2.3 Cells

The source of the ovarian cancer cell linesOVCAR-3, SKOV-3and Caov-3 and breast cancer cell line SKBr-3 has been described previously (18). These cells were cultured in RPMI medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. All cell lines were frozen at early passages and used for <10 weeks in continuous culture.

2.4 Measurement of IL-6 production by ELISA

Clinical specimens and culture supernatants of cell lines treated without or with LPA or other stimuli were collected and analyzed for measuring IL-6 concentrations by ELISA using the human IL-6 Quantikine ELISA kit (R&D Systems). Concentrations of IL-6 in culture supernatants and clinical samples were calculated by comparing the absorbance of samples to standard curves.

2.5 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 MgCl2, 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(vinylidenedifluoride)], and immunoblotted with antibodies following the protocols provided by the manufacturers. Immunocomplexes were visualized with an enhanced chemiluminescence detection kit (Amersham Biosciences) using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA).

2.6 Plasmids The C/EBP-β, liver-enriched transcriptional activator protein 1 (LAP1) and LAP2 expression vectors were kindly provided by Dr. L. Sealy (Vanderbilt University School of Medicine) [53, 54]]. The expression of C/EBP-β from these vectors in transfected cells was confirmed by immunoblotting. The dominant negative form of C/EBP-β, LIP (liver-enriched inhibitory protein), [55] was cloned into pcDNA3.1 by RTPCR amplification of a 444 bp cDNA fragment of C/EBP-β from Caov-3 cells. The structure of pcDNA3-LIP was confirmed by automatic sequencing and immunoblotting analysis of expression of the short, truncated form of C/EBP-β (21 kD) [54] in transfected cells.

2.7 Transient transfection and luciferase assays Ovarian cancer cell lines were seeded in 6-well plates and grown to 30-40 % confluence before transfection with the luciferase vectors using Fugene 6 (Roche) or TransIT-TKO (Mirus Bio Corp., Madison, WI) according to the instructions of the manufacturers. About 48 hours after transfection, the cells were starved for 24-36 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).

RESULTS

3.1 IL-6 levels are elevated in plasma and ascites of ovarian cancer patients.

IL-6 is a pro-inflammatory molecule that is an important mediator of ovarian cancer. To explore the role of IL-6 in ovarian cancer, we set out to determine IL-6 levels in ovarian cancer patient samples by ELISA. As shown in Figure 2, normal controls displayed consistently low levels of IL-6 (<25 pg/ml) in their plasma samples. However, the concentrations of IL-6 in ovarian cancer patients varied remarkably. About 33% of patients (27 out of 82) showed a dramatic increase in IL-6 concentrations in the plasma, with the remaining patients having levels similar to those of normal individuals.

To track the origin of the increased IL-6 levels in ovarian cancer patients, we compared IL-6 levels in the plasma and ascites of ovarian cancer patients. We analyzed IL-6 levels in the plasma and matched ascites samples from 21 ovarian cancer patients. As shown in Fig 3, IL-6 levels in ascites were consistently higher than the matched plasma concentrations. For example, the IL-6 levels in patient 10 was about 100 pg/mL in the plasma, but reached 13,000 pg/mL in the ascites. These observations suggest that IL-6 is produced in the ascites, likely by ovarian cancer cells, and migrate from the peritoneal cavity to the blood circulation.

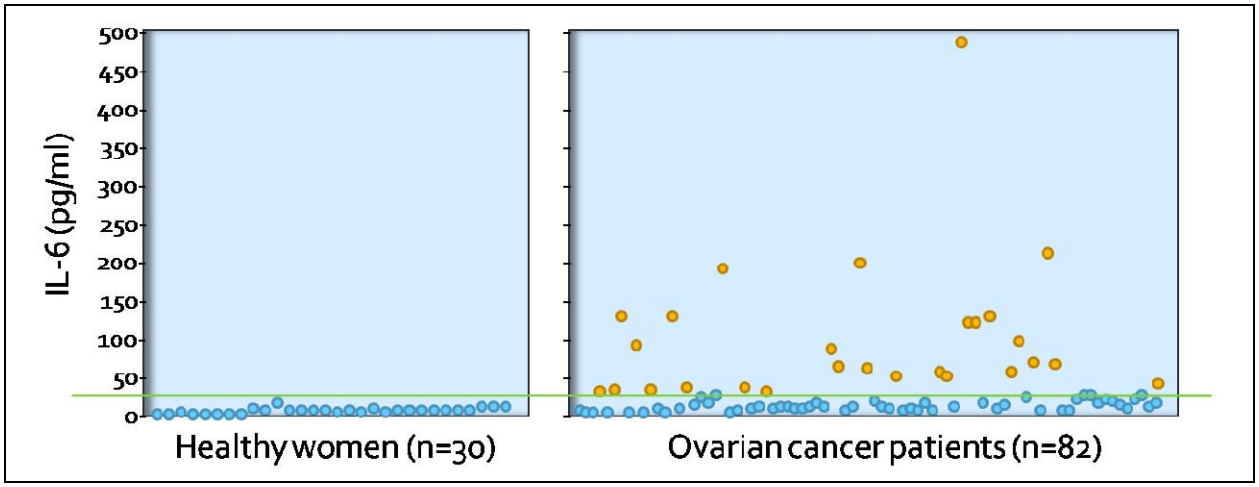

Figure 2. IL-6 in the plasma of ovarian cancer patients. The IL-6 levels (pg/mL) in the plasma of normal female volunteers and in the plasma of ovarian cancer patients were quantified by ELISA as described in Materials and Methods. The plasma IL-6 levels of normal controls (n=30) were compared with those of ovarian cancer patients (n=82).

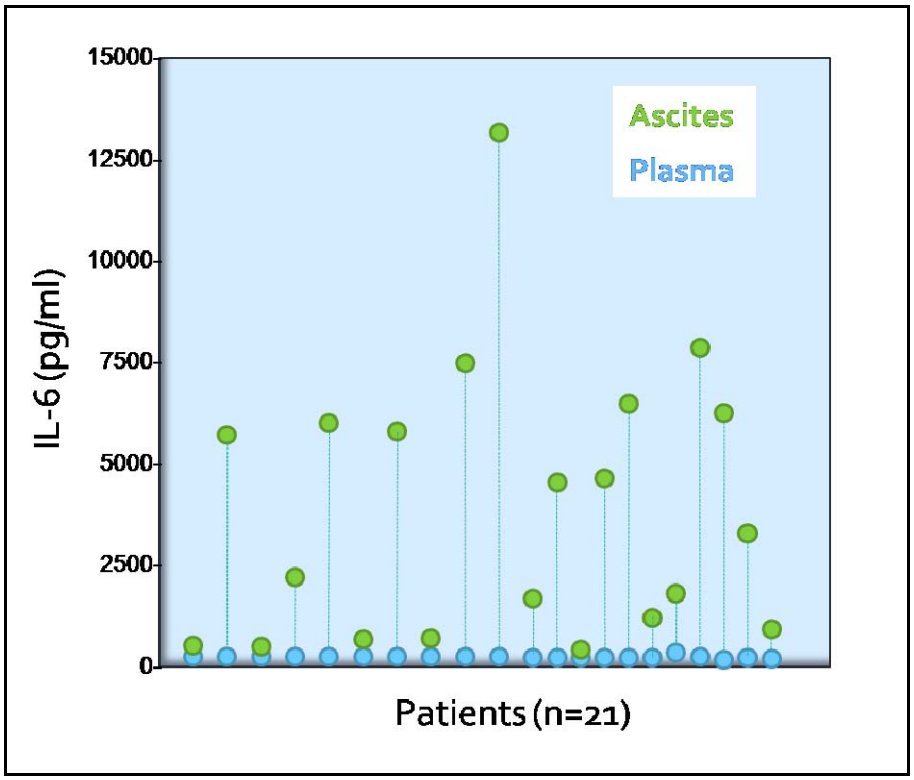

Figure 3. . IL-6 levels in the plasma and ascites of ovarian cancer patients (matched samples). IL-6 concentrations in plasma and matched ascites were determined by ELISA in 21 ovarian cancer patients from whom both plasma and ascites were available.

3.2 IL-6 expression by ovarian cancer cells is induced by serum.

We then determined whether ovarian cancer cells indeed generate IL-6. We quantified IL-6 in conditioned medium of ovarian cancer cell lines. Ovarian cancer cell lines were cultured to approximately 60% confluence, starved in serum-free medium for 24 hours with conditioned medium collected at 12-hour intervals for ELISA analysis. The results showed that IL-6 concentrations in conditioned medium decreased rapidly when the cells were starved in serum-free conditions. By 12 hours, IL-6 levels in the medium were reduced to < 40% of the original values at time 0. By 24 hours of incubation in serum-free medium, IL-6 concentration was further reduced to less than 15% of the originalIL-6 level. The results indicate that IL-6 is not constitutively expressed, but its expression or release by ovarian cancer cells is induced by serum.

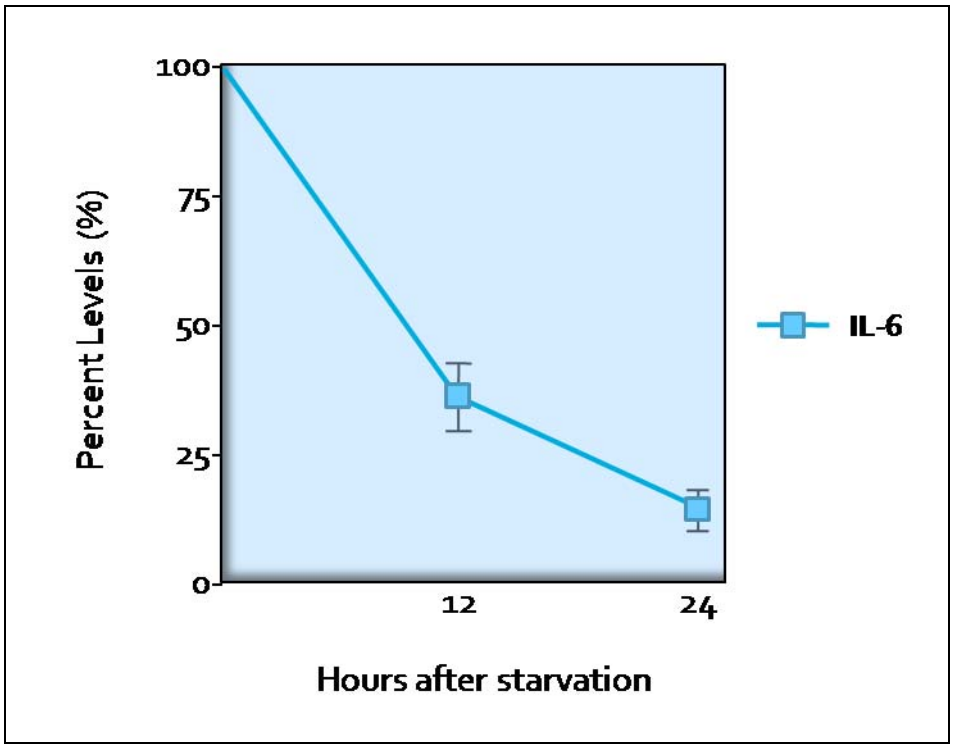

Figure 4. Serum-dependent production of IL-6 by ovarian cancer cells. Ovcar-3 cells were cultured in fresh complete medium for 12 hours (time 0) and then switched to serum-free medium for 24 hours with the conditioned medium collected at 12-hour intervals for ELISA. The IL-6 concentration at time 0 was defined as 100% relative to those at 12 or 24 hours after the beginning of starvation.

3.3 LPA stimulates production of IL-6 in ovarian cancer cells.

To identify the factors in serum that were responsible for inducing IL-6 expression in ovarian cancer cells, we assessed the effects of a number of peptide growth factors as well as lysophospholipid growth factors present in serum. The peptide growth factors assessed were insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), and hepatocyte growth factor (HGF). However, none of these peptide growth factors induced IL-6 production significantly. On the contrary, LPA was effective in stimulation of IL-6 expression. At 10 μM, different species of LPA including 14:0, 16:0 and 18:1 LPA were all potent inducers of IL-6 production. Phosphatidic acid (PA, 16:0) demonstrated a much weaker stimulatory effect on IL-6 production. PA might trigger IL-6 production through conversion to LPA in culture. FBS strongly induced IL-6 expression likely because it contained multiple growth factors including LPA. These observations suggest that the stimulation of IL-6 production in ovarian cancer cells is likely mediated by LPA rather than peptide growth factors.

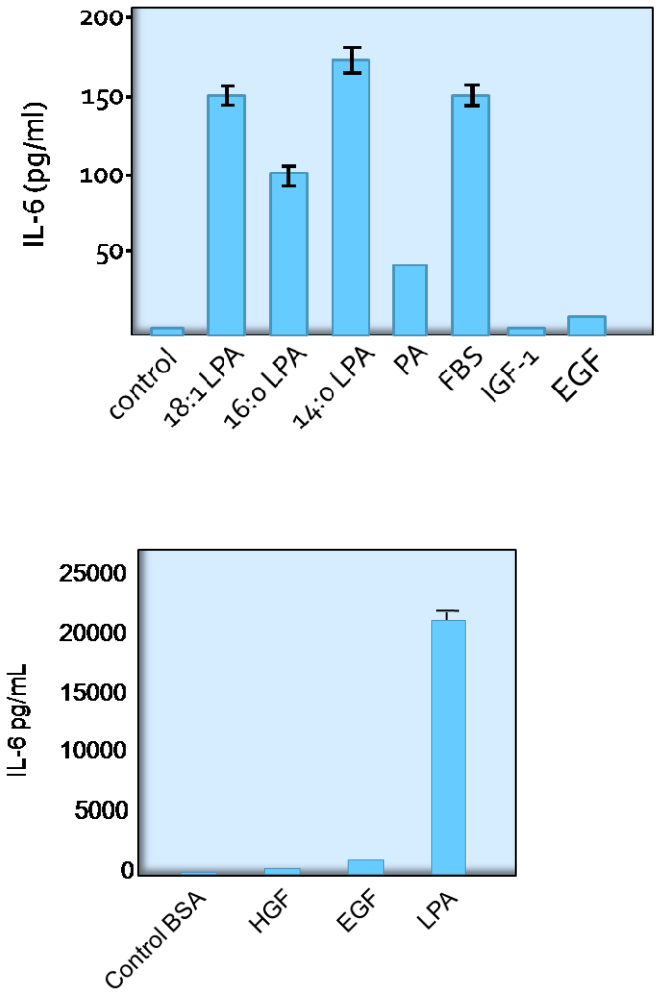

Figure 5. Comparison of the effects of LPA, FBS and prototype peptide growth factors on IL-6 production in ovarian cancer cell lines. After starvation in serum-free medium for 24 h, OVCAR-3 (top) or Caov-3 (bottom) cells were incubated for 16 h with vehicle (control), 18:1, 16:0, or 14:0 LPA (10 μM), 16:0 PA (10 μM), FBS (5%), IGF-1 (50 ng/ml), EGF (25 ng/ml) or HGF (20 ng/ml). IL-6 levels in the culture supernatants of treated cells were quantified by ELISA.

Subsequently, we focused on the effect of LPA on the expression of IL-6 in ovarian cancer cells. In the cell line OVCAR-3, LPA stimulated production of IL-6 in a dose-dependent manner. Overnight incubation of OVAR-3 cells with 10 uM of LPA led to multi-fold increases in IL-6 concentrations in the culture supernatants as analyzed by ELISA (Fig. 6). In contrast, the breast cancer cell line SKBr-3 had minimal IL-6 production. However, this does not indicate that breast cancer cell lines are unresponsive to LPA. In fact, most breast cancer cell lines did respond to LPA with IL-6 production (data not shown). SKBr-3 lacks functional levels of LPA receptors as we and others reported previously [56, 57].

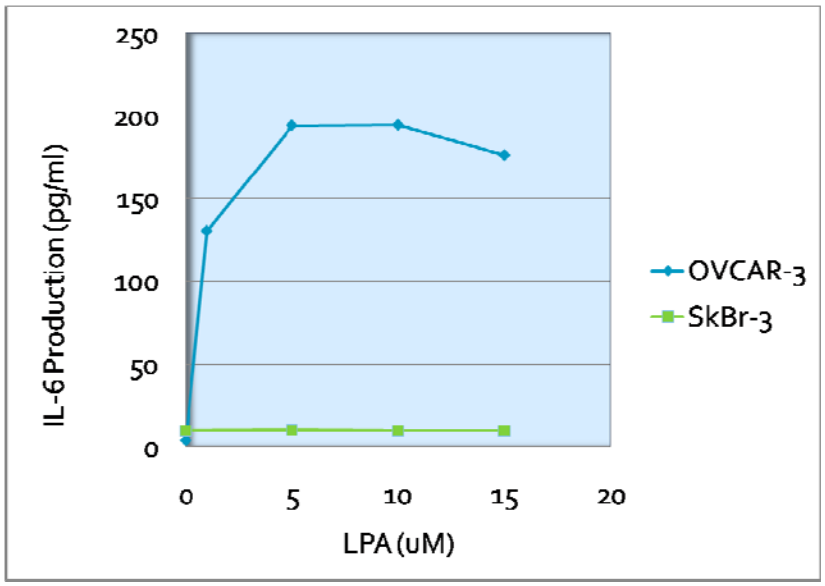

Figure 6. LPA stimulation of IL-6 production in ovarian cancer cell lines. After starvation in serum-free medium for 24 h, OVCAR-3 cells (in 6-well plates) were incubated with increasing concentrations of LPA (18:1) for 14–16 h. Culture supernatants were collected and assayed for IL-6 levels by ELISA analysis. Curve is plotted as amounts of IL-6 (pg) produced by 5 x 10^5 cells treated with indicated concentrations (µM) of LPA.

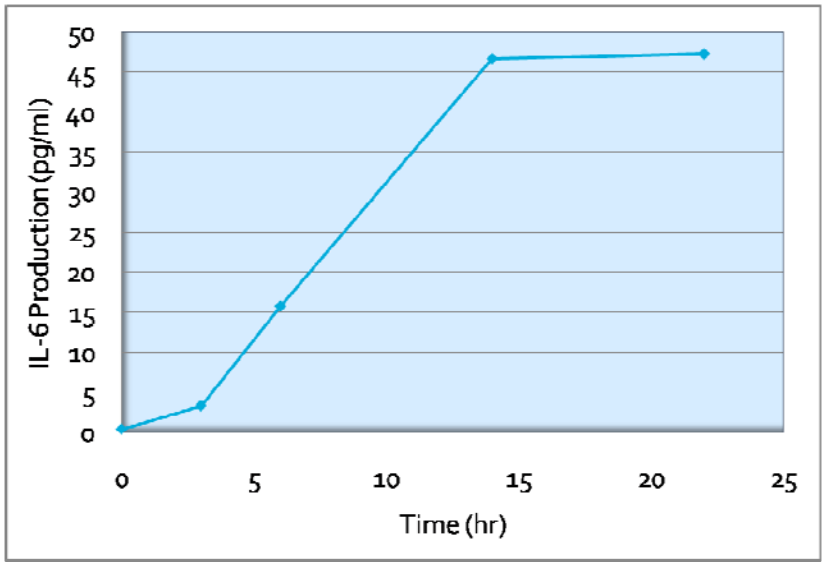

Figure 7. Time-dependent induction of IL-6 by LPA. OVCAR-3 cells were starved and stimulated with LPA (10 µM) for the indicated periods of time (hours). IL-6 levels in culture supernatants collected at the indicated time points were determined by ELISA.

3.4 LPA activates the IL-6 gene promoter

To test if LPA induces the transcriptional activation of the IL-6 gene, we characterized the IL-6 promoter by the luciferase reporter assay. Ovarian cancer cell lines were transfected with the plasmid pIL-6-Luc in which the luciferase reporter gene was placed under the control of the IL-6 gene promoter. In all ovarian cancer cell lines tested, treatment of the transfected cells with LPA resulted in marked increases in luciferase activity (Fig. 8), confirming that LPA indeed triggers transcriptional activation of the IL-6 gene promoter.

We then analyzed the IL-6 gene promoter by making mutation in binding sites for NF-κB or C/EBP. The point mutations eliminate the ability of the promoter to bind to their corresponding transcription factors. Mutation of the unique NF-κB site led to an approximately 75% loss of LPA-stimulated luciferase activity. This indicates that the NF-κB site is critical for activation of the IL-6 promoter by LPA.

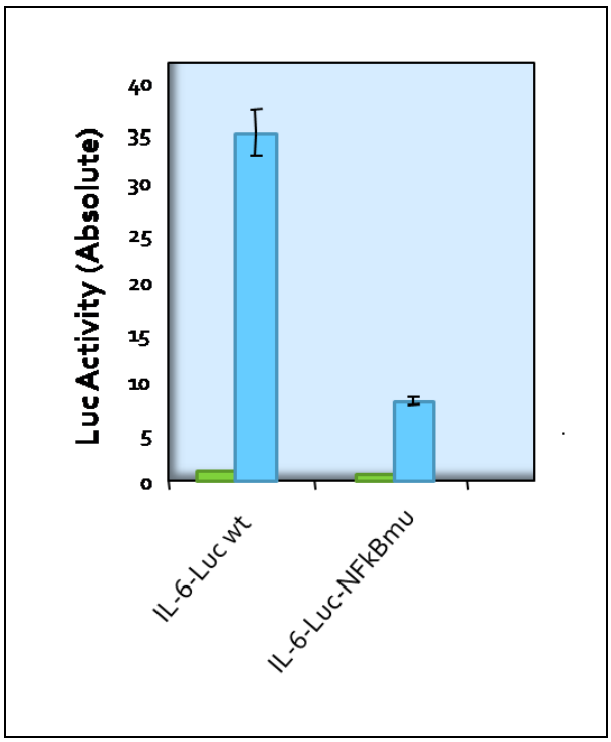

Fig. 8. LPA activation of the IL-6 gene promoter: Involvement of NF-κB. Caov-3 cells were transfected with pIL-6-Luc or with pIL-6-Luc-NFκBmu (NF-κB mutant luciferase reporter). The transfected cells were starved and stimulated with 10 μM LPA for 6 hours. The luciferase activity (arbitrary unit) in cell lysates was determined by luciferase assay as described in Materials and Methods.

To further examine the role of the NF-κB binding site in the activation of the IL-6 promoter, we used siRNA to down-regulate the expression of Bcl10. Bcl10 is a gene that encodes a protein involved in GPCR-mediated activation of NF-κB. Bcl10 protein forms a complex with MALT1 and CARMA to mediate antigen receptor-induced NF-κB in lymphocytes. This complex activates the IKK complex through ubiquitination of the IKKγ/NEMO subunit of the IKK complex in antigen receptor signaling pathways (Fig. 9, [58]). Similarly, the Bcl10/MALT1/CARMA signalsome has been recently found to be essential for NF-κB activation induced by GPCR including LPA receptors (Fig. 9). Thus LPA-induced NF-κB activation depends on intact Bcl10. Upon down-regulating the expression of Bcl10, we observed a significant decrease in LPA-induced IL-6 production in Caov-3 cells as shown in Fig. 10 reaffirming the importance of NF-κB in activation of IL-6 production by LPA.

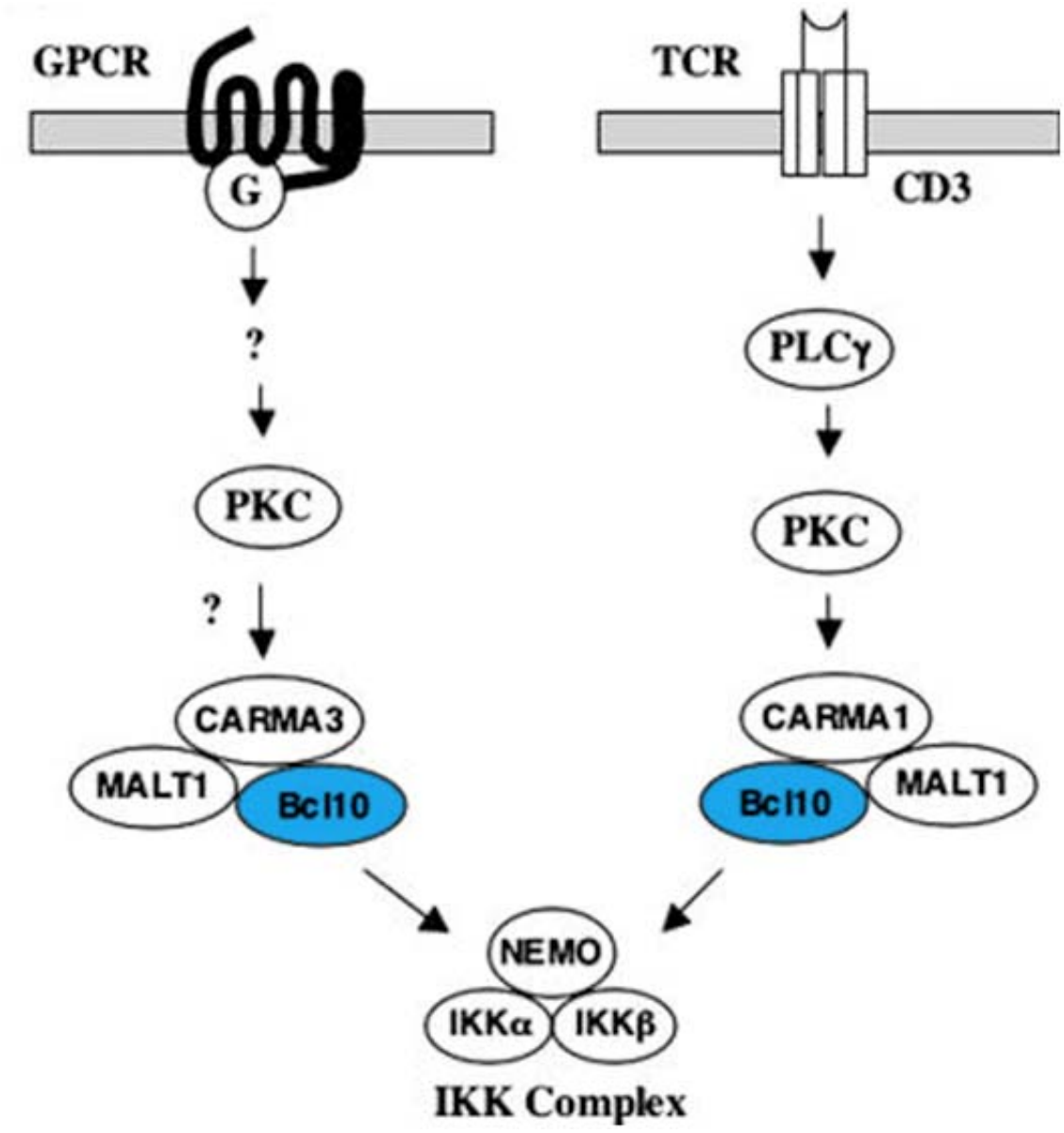

Figure 9. A schematic model of GPCR-induced and T cell receptor (TCR)-induced signaling cascades (modified from [58]). In the GPCR pathway, proximal signaling events lead to activation of PKC, which, in turn, may regulate CARMA3 and Bcl10. The activated CARMA3 and Bcl10 may directly or indirectly regulate the IKK complex. In contrast, in the TCR pathway, PKC phosphorylates CARMA1, which induces the formation of the CARMA1–Bcl10–MALT1 complex, leading to activation of the IKK complex.

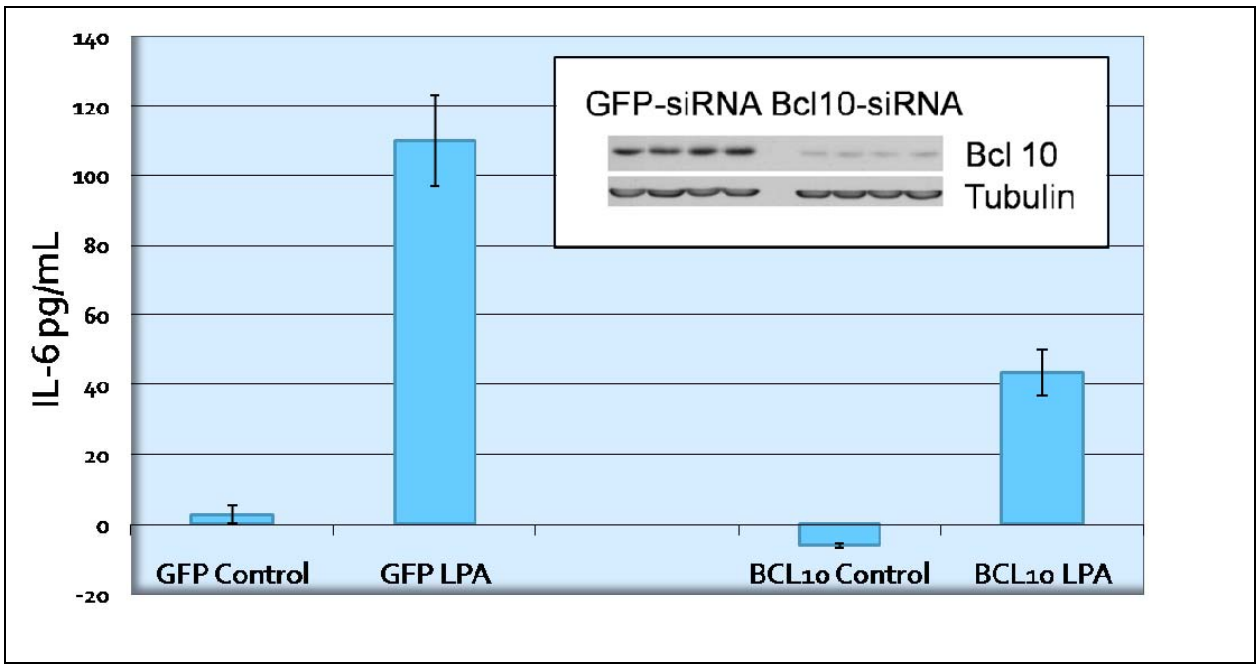

Figure 10. Inhibition of LPA-induced IL-6 production by downregulation of Bcl10. Bcl10 expression in SKOV-3 cells was silenced by siRNA. The cells treated with control siRNA (GFP siRNA) or Bcl10 siRNA were starved and stimulated with 10 μM LPA for 16 hours. IL-6 levels in culture supernatants were determined by ELISA. The downregulation of Bcl10 by siRNA was confirmed by western blotting.

C/EBP stands for CCAAT/enhancer binding proteins and is a family of basic region-leucine zipper (bZIP) class of transcription factors that play essential roles in many physiological and pathological processes such as cellular differentiation and inflammation. There are two potential C/EBP binding sites on the IL-6 gene promoter. We transfected Caov-3 cells with wild type or C/EBP mutated IL-6 promoter luciferase reporter. The transfected cells were starved and then treated with LPA (10 μM, 6 hr) before luciferase assay. Compared to the wild type reporter, the C/EBP mutant showed a marked decrease in luciferase activity in response to LPA (Fig. 11), indicating that the C/EBP binding site is also involved in LPA activation of the IL-6 promoter.

We further tested the importance of C/EBP binding site on the IL-6 promoter by using a dominant negative form of C/EBP called LIP. LIP (liver-enriched inhibitory protein) is a truncated isoform of C/EBPβ. It lacks a transactivation domain, and serves as a transcriptional repressor. pcDNA3-LIP or pcDNA3 was co-transfected into Caov-3 cells along with pIL-6-Luc. Co-expression of LIP strongly decreased LPA-induced luciferase activity in these cells as demonstrated in Fig. 12. The observation further confirms implication of C/EBP in LPA stimulation of IL-6 promoter and gene expression.

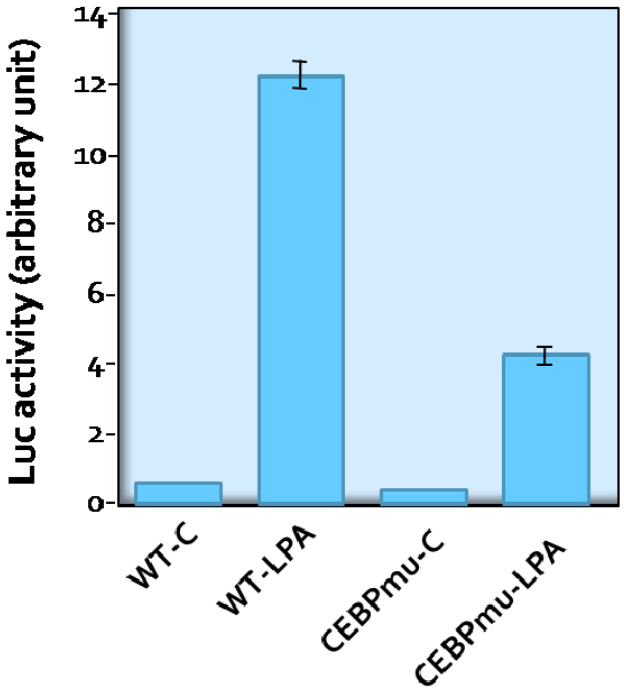

Fig. 11. LPA activation of the IL-6 gene promoter: involvement of C/EBP. Caov-3 cells were transfected with pIL-6-Luc or with pIL-6-Luc-C/EBPmu (C/EBP mutant luciferase reporter). The transfected cells were starved and stimulated with 10 μM LPA for 6 hours. The luciferase activity (arbitrary unit) in cell lysates was determined by luciferase assay as described in Materials and Methods.

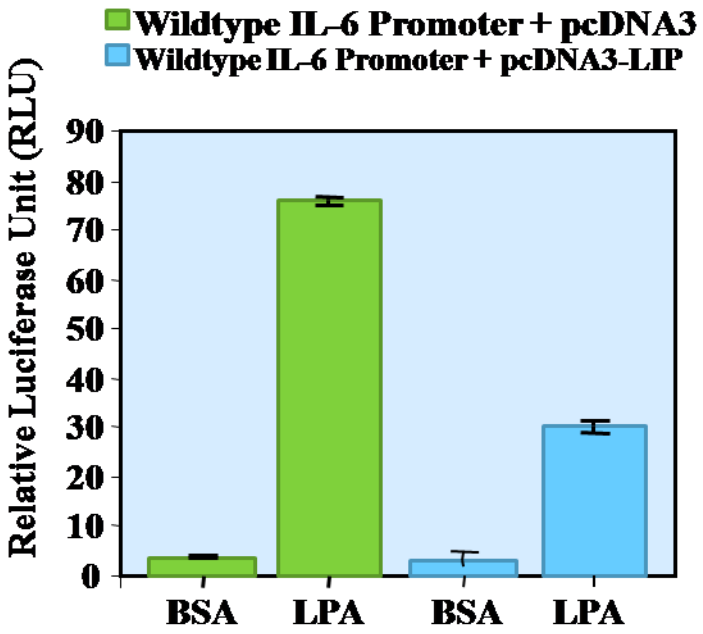

Figure 12. Inhibition of the IL-6 promoter activity by expression of LIP, a dominant negative form of C/EBP. Caov-3 cells were co-transfected with pcDNA3 or pCDNA3-LIP along with the pIL-6-Luc reporter. The transfected cells were starved and then stimulated with LPA (10 μM) for 6 hours and assayed for luciferase activity.

3.5 LPA induces Stat3 phosphorylation

To understand the biological significance of LPA-mediated increase in IL-6 levels in ovarian cancer, we examined one of the pathways downstream of the IL-6 receptor. Although IL-6 triggers activation of numerous signaling cascades such as Ras/MAPK and PI3K-Akt, we focused on the more characteristic Jak/Stat pathway. As mentioned before, Stat-3 is phosphorylated by Jaks before it dimerizes and translocates into the nucleus to regulate gene transcription. We stimulated ovarian cancer cells with LPA for different time durations and ran a western blot to determine its effect on Stat-3 phosphorylation. The results showed that Stat3 was phosphorylated on the serine residue S727 as early as 10 minutes after stimulation with LPA, but the phosphorylation at the tyrosine residue Y705 reached significant levels after 2-4 hours of stimulation with LPA, an observation compatible with an effect secondary to LPA stimulation.

Since IL-6 is a prototype inducer of Stat phosphorylation and activation, we tested if IL-6 was involved in LPA-induced tyrosine phosphorylation of Stat-3 via an autocrine loop. We utilized an IL-6 neutralizing antibody to prevent bioactivity of IL-6. Western blot analysis showed that the neutralizing IL-6 antibody at 5 μg/ml only slightly attenuated Stat-3 phosphorylation at Y705. We then examined expression of the IL-6 receptor in ovarian cancer cell lines. Western blot analysis showed a moderate expression of the IL-6 receptor in Caov-3, OVCAR-3 and SKOV-3 ovarian cancer cell lines. These results indicate that ovarian cancer cells express both IL-6 and its receptor. However, LPA-

induced Stat-3 phosphorylation seems independent of this autocrine loop. LPA may stimulate Stat3 tyrosine phosphorylation directly or through other secondary mediators rather than IL-6.

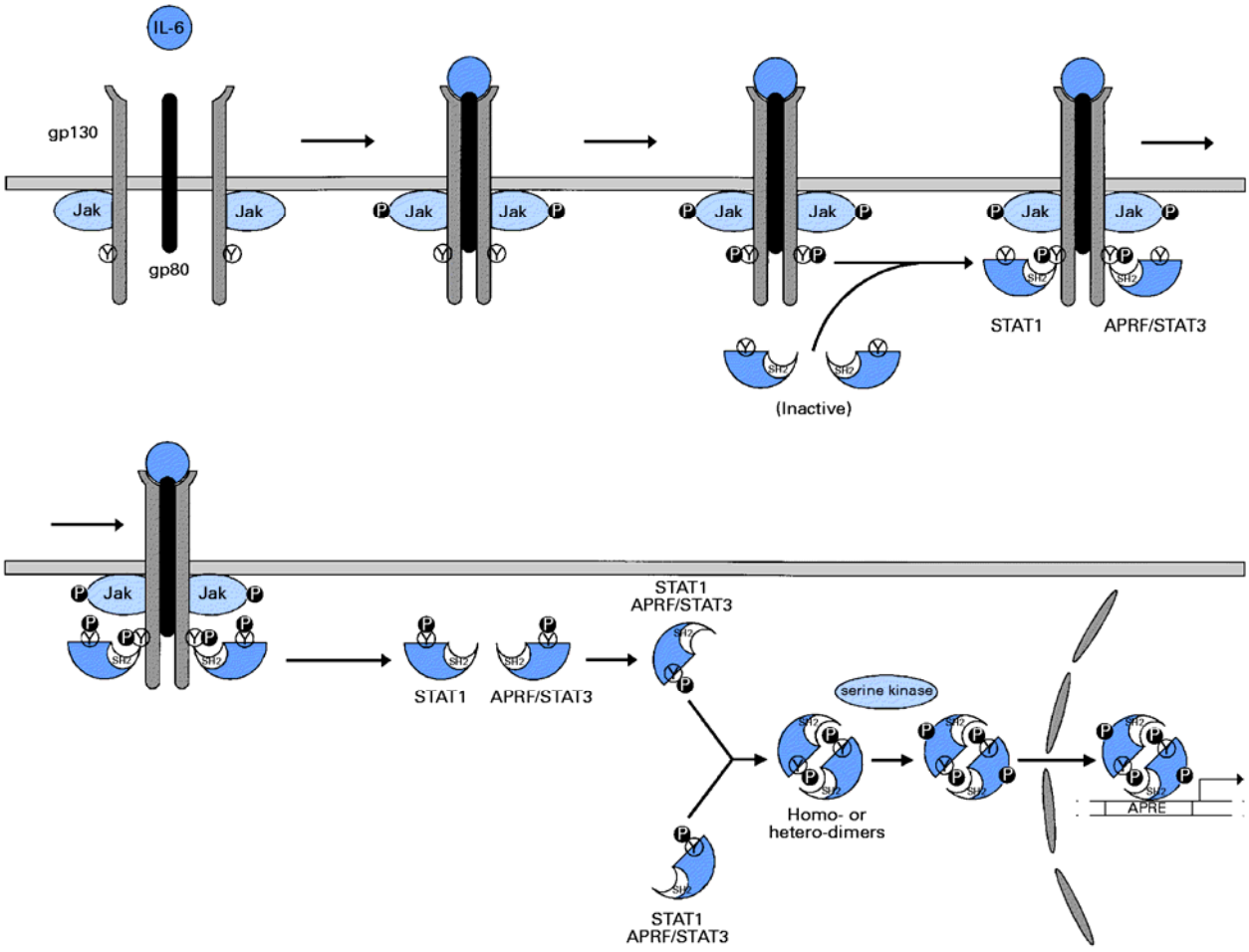

Figure 13. IL-6 signaling to Stat3 phosphorylation and activation. IL-6 binding induces receptor dimerization, activating the associated Jaks, which phosphorylate themselves and the receptor. The phosphorylated sites on the receptor and Jaks serve as docking sites for the SH2-containing Stats, such as Stat3, and for SH2-containing proteins and adaptors that link the receptor to MAP kinase, PI3 Kinase/Akt and other cellular pathways. Receptor-bound Stats phosphorylated by Jaks dimerize and translocate into the nucleus to regulate target gene transcription. Image modified from [59].

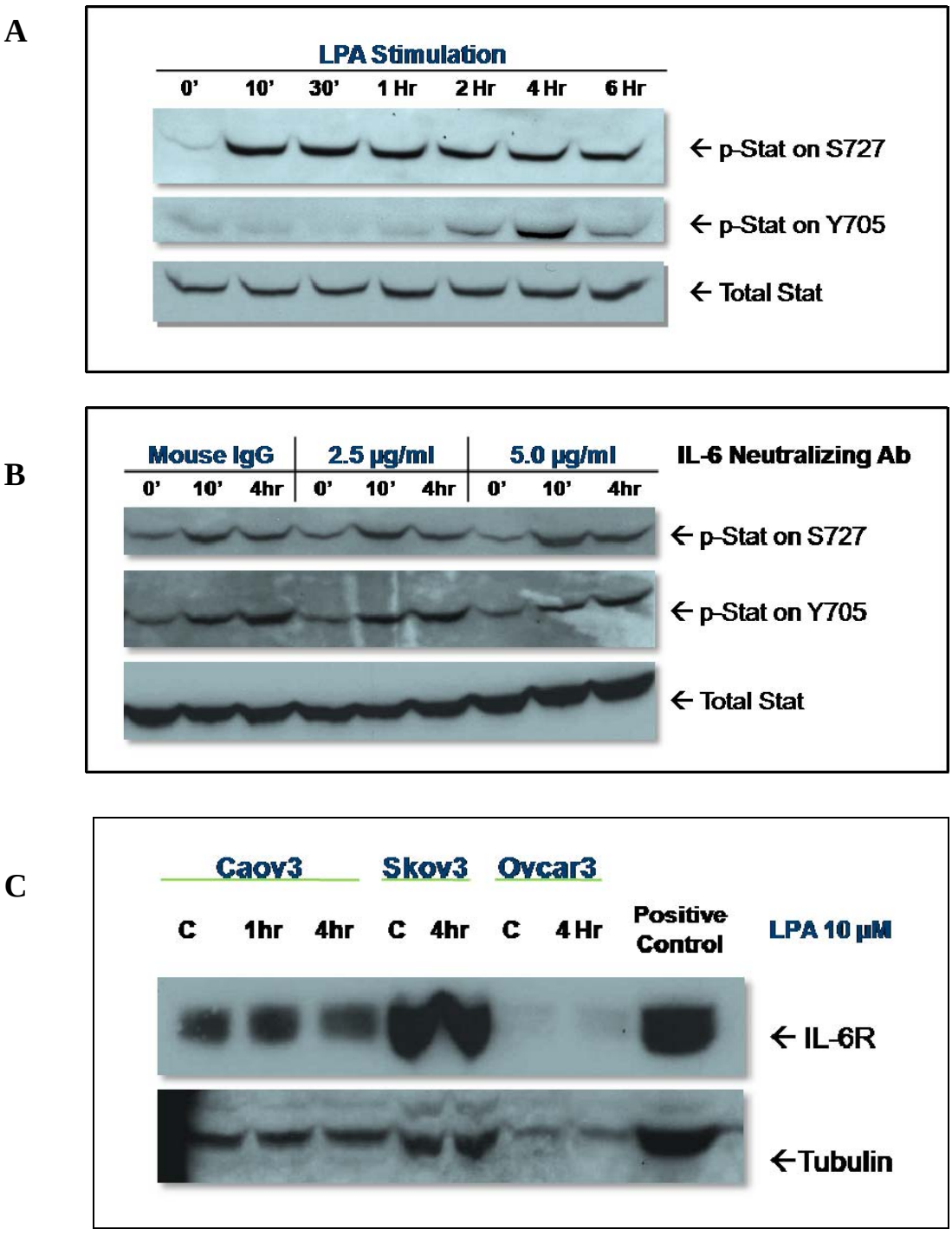

Figure 14. LPA induction of Stat3 phosphorylation and activation independent of IL-6 autocrine loop. A, Serum-starved Caov-3 cells were stimulated with 10 µM LPA for the indicated periods of time and analyzed for phosphorylated Stat by western blotting. **B.** Caov-3 cells treated with LPA for 10 min or for 4 hours in the presence of indicated doses of IL-6 neutralizing antibody. Stat3 tyrosine phosphorylation was analyzed with western blotting. **C.** Expression of the IL-6 receptor (IL-6R, gp80) in Caov-3, Skov-3, and Ovcar-3 cells were analyzed with western blotting.

DISCUSSION

Since IL-6 is implicated in ovarian cancer progression and prognosis [51] , it is important to understand the regulatory mechanisms that underlie over expression of this cytokine in ovarian cancer. In the current study, we demonstrate that IL-6 is a major target gene of LPA in ovarian cancer cells by analyzing the transcriptional changes in response to LPA stimulation. We showed that the high levels of IL-6 in ascites may be due to their synthesis and secretion by ovarian tumor cells under impact of LPA. Our finding is of particular interest as LPA has been identified as an important growth factor for ovarian cancer. Its concentration is markedly and consistently elevated in the ascites of ovarian cancer patients, ranging from 1 to 80 μmol/L [59-61], providing an LPA-rich microenvironment for ovarian tumor cells. The high levels of IL-6 in ascites compared to the plasma of ovarian cancer patients imply that IL-6 is synthesized by ovarian tumor cells in ascites and migrate to the bloodstream, forming a gradient between ascites and blood circulation of ovarian cancer patients.

In an effort to understand how IL-6 production is up regulated in ovarian cancer, we demonstrated that serum is a potent stimulus of IL-6 production in ovarian cancer cell lines. The ability of serum to stimulate IL-6 generation was not reproduced by peptide growth factors such as IGF-1, EGF or HGF. Instead, different species of LPA are strong stimuli of IL-6 production in ovarian cancer cells. The inability of various peptide growth factors to induce generation of IL-6 in ovarian cancer cells underscores the importance of LPA and other lipid mediators in the regulation of this cytokine by serum. Serum contains LPA and other phospholipid factors in addition to the well-defined peptide growth factors.

Because serum contains significant concentrations of LPA and LPA-producing enzyme ATX, it is plausible that IL-6 production in ovarian cancer patients is an outcome of LPA which is present at elevated levels in the blood and ascites of ovarian cancer patients [1, 3].

In this study, we also sought to determine the mechanism by which LPA increases IL-6 production. Recent studies from our group and from other laboratories indicate that LPA may mediate oncogenesis through regulation of gene expression [46, 57]. Our results indicate that LPA stimulates transcriptional activation of IL-6 involving two prominent transcription factors, NF-kB and C/EBP. NF-kB seems to be essential for LPA stimulation of IL-6 production as mutation of the NF-kB site on the promoter almost eliminated the response to LPA. Our results also showed an important role of Bcl10 in activation of NF-kB and IL-6 production by LPA. Recent studies from several independent groups showed the intermediary role of Bcl10 in GPCR-mediated activation of NF-kB [58, 62]. Indeed, when we down regulated Bcl10 expression, LPA-induced IL-6 production was dramatically decreased.

The C/EBP transcription factor regulates expression of many target genes including those of cytokines. However, C/EBP does not play a significant role in LPA-stimulated IL-8 expression [57]. Recently, studies from our lab found that C/EBP is the key transcription factor responsible for LPA up regulation of the Cox-2 gene in ovarian cancer cells. C/EBP is activated through phosphorylation induced by LPA [46]. In this study, we extend the role of C/EBP to LPA-induced IL-6 gene expression. The link of C/EBP to the biological functions of LPA is an interesting observation as C/EBP is over expressed in ovarian cancer [63]. The results from current study suggests that C/EBP may contribute to ovarian

oncogenesis through its participation in regulation of cancer-related gene expression by LPA.

In the last section of this thesis, we showed that LPA induced Stat3 phosphorylation at both serine and tyrosine residues. Since Y705 phosphorylation did not occur immediately after exposure to LPA, we speculated that the delayed response to LPA might be derived from bioactivity of IL-6 via an autocrine loop. We took advantage of an IL-6 neutralizing antibody commonly used by others to block IL-6 activity [64]. Although ovarian cancer cell lines expressed the IL-6 receptor, the IL-6 antibody failed to significantly prevent LPA-induced Stat-3 phosphorylation at Y-705. Thus the results indicate that LPA stimulates Stat-3 directly or through secondary mediator other than IL-6. Future studies targeting other signaling cascades should help determine exactly how LPA induces Stat-3 activation independently of IL-6.

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