MECHANISMS OF GROWTH FACTOR-INDUCED LYSOPHOSPHATIDIC ACID PRODUCTION IN OVARIAN CANCER CELLS

by

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of ASHLEY CAMERON JONES find it satisfactory and recommend that it be accepted.

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Abstract

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Lysophosphatidic acid (LPA) refers to a family of small lipid mediators involved in diverse cellular processes. LPA binds to G-protein coupled receptors to induce cellular effects that include proliferation, angiogenesis, and migration and metastasis of tumor cells. Levels of LPA have been shown to be increased in the acites fluid surrounding ovarian tumors. Several enzymes are potentially involved in the production and metabolism of LPA, including phospholipase D (PLD), and autotaxin (ATX). However, the pathways and mechanisms that regulate LPA production are poorly understood. The hypothesis addressed in this dissertation was that agonist-induced LPA production involves PLD2. To investigate this hypothesis, we examined agonist-induced lysophosphatidic acid production in two ovarian cancer cell lines, OVCAR3 and SKOV3. The results show that epidermal growth factor (EGF) can stimulate LPA production by the cells within 30 minutes of stimulation. EGFR inhibition blocks both EGF- and LPA-induced LPA production in both cell lines, and the LPA receptor antagonist

Ki16425 inhibits both LPA and EGF-induced LPA production in SKOV3 cells. Autotaxin (ATX) can produce LPA extracellularly. However, we show that endogenous ATX activity does not account for the rapid agonist-induced increase in LPA in the medium of OVCAR3 or SKOV3 cells. EGF and LPA both stimulate PLD2 activity in ovarian cancer cells. Over-expression of PLD2 increases PLD activity in OVCAR3 cells and membrane preparations. PLD2 over-expression also increases basal levels of LPA production. Small interfering RNAs (siRNAs) were used to down-regulate PLD2 activity in OVCAR3 cells. These siRNAs blocked both membrane PLD activity and EGF-induced LPA production in OVCAR3 cells. Together these results suggest that receptor crosstalk and activation of PLD2 are necessary for agonist-induced LPA production in ovarian cancer cells.

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KEY TO ABBREVIATIONS

5-LO:	5-lipoxygenase
AC:	adenylyl cyclase
AGK:	acylglycerol kinase
ARF:	small GTP-binding protein
Akt/PKB:	protein kinase B; kinase activated via PI3K
ATX:	autotaxin
B-MG:	BODIPY-monoacylglycerol
B-LPA:	BODIPY-lysophosphatidic acid
BRCA:	breast and ovarian cancer susceptibility genes
BPC:	BODIPY-phosphatidylcholine
B-LPC:	BODIPY-lysophosphatidylcholine
C-Src:	intracellular tyrosine kinase; cellular version of Rous sarcoma virus tyrosine kinase
DMEM:	Dulbecco's modified Eagle's medium
EDG:	endothelial differentiation gene
Erk:	extracellular signal-related kinase
EGF:	epidermal growth factor
EGFR:	epidermal growth factor receptor
EGTA:	ethylene glycol bis tetraacetic acid
FAK:	focal adhesion kinase
GPCR:	G protein-coupled receptor
GTP:	guanosine triphosphate

HB-EGF:	heparin-binding epidermal growth factor
Her3:	member of the epidermal growth factor receptor family
Her4:	member of the epidermal growth factor receptor family
Her2/Neu:	members of the epidermal growth factor receptor family
IGF-1 BP:	insulin-like growth factor binding protein
LF2000:	lipofectamine 2000
LPA:	lysophosphatidic acid
LPAR:	lysophosphatidic acid receptor
LPC:	lysophosphatidylcholine
LPP:	lipid phosphate phosphatase
LPAAT:	lysophophosphatidic acid acyl transferase
Lyso-PLD:	lyso-phospholipase D
LT:	leukotriene
LTR:	leukotriene receptor
MAPK:	mitogen activated protein kinase
MEK:	MAPK/Erk kinase; dual function kinase that activates Erks
MMP:	matrix metalloprotease
PA:	phosphatidic acid
PAGE:	polyacrylamide gel electrophoresis
PBt:	phosphatidylbutanol
PC:	phosphatidylcholine
PDGFR:	platelet derived growth factor receptor
PEt:	phosphatidylethanol

PH:	pleckstrin homology
PIP2:	phosphatidylinositol bisphosphate
PI3K:	phosphoinsositide-3 kinase
PKC:	protein kinase C
PLA:	phospholipase A
PLC:	phospholipase C
PLD:	phospholipase D
PMA:	phorbol 12-myristate 13-acetate
PtdOH:	phosphatidyl alcohol
PTX:	pertussis toxin
Raf:	protein serine/threonine kinase that activates MEK
Ras:	small GTP-binding protein that activates Raf
Rho:	small GTP-binding protein involved in cytoskeletal reorganization
RT-PCR:	reverse transcriptase-polymerase chain reaction
SDS:	sodium dodecyl sulphate
SEM:	standard error of measurement
siRNA:	small interfering RNA
TGFα:	transforming growth factor alpha
TLC:	thin layer chromatography
VEGF:	vascular endothelial growth factor

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DEDICATION

I would like to dedicate this work to my family and friends. The love, support, and encouragement that they have given me over the years has made all this possible. Their words of encouragement have helped me when I have needed it the most.

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CHAPTER I

GENERAL INTRODUCTION

1.1 LYSOPHOSPHATIDIC ACID STRUCTURE-ACTIVITY RELATIONSHIP

Lysophosphatidic acid (LPA) refers to a family of small biologically active phospholipids. LPA in the general form, 1-*O*-acyl-2-hydroxy-*sn*-glyceryl-3-phosphate, is composed of a glycerol backbone with several possible side chains at the 1- or 2- positions, and a phosphate group at the 3-position. The substituents vary in length, composition, and linkage to the glycerol backbone (43). Side chains can vary in length from short chains (C6-C8) to longer chains (C20-C24) with differing degrees of saturation. The side chain length and degree of saturation are used to name the different species of LPA.

Several different saturated LPAs have been identified in mammalian plasma: hexanoyl (6:0), myristoyl (14:0), palmitoyl (16:0), and stearoyl (18:0) (44), as well as unsaturated LPAs: oleoyl (18:1), linoleoyl (18:2), and arachidonoyl (20:4) (44, 138). Chain length and linkage are important not only for species identification, but for structure-activity relationships as well. The sn-1 or sn-2 linkages to the backbone are by three different known bonds: ester, alkyl, or alkenyl ether (137). The acyl subclass can be further divided to include substituents at the sn-1 (1-acyl-LPAs) and sn-2 positions (2-acyl-LPAs).

Studies have been performed to determine the relationships between structure and function for different species of LPA. The structure-activity relationship of LPA for platelet aggregation differs slightly from that for mitogenic responses in fibroblasts (88, 132). LPA-mediated cardiovascular responses can be further divided into two categories, smooth muscle and platelet. Poly-unsaturated acyl LPAs have a higher affinity for receptors on smooth muscle

cells than do saturated acyl or alkyl-ether linked LPAs, while alkyl ether-linked LPAs have a higher affinity for platelets (128). Acyl-linked LPAs have proven to be the most potent at inducing mitogenesis. The rank order for efficacy of LPA species with respect to mitogenesis is 18:1=16:0>14:0>12:0>10:0, indicating that unsaturation as well as fatty acid chain length are important for mitogenic activity (132). Oleoyl (18:1)-LPA is one of the most abundant LPAs in mammalian cells, and is the most potent and efficacious in many biological response assays.

1.2 LYSOPHOSPHATIDIC ACID RECEPTORS AND SIGNALING

LPA has been shown to activate three G-protein coupled receptors (GPCRs). LPA binds to and activates members of the endothelial differentiation gene family, Edg -2, -4 and -7, now known as LPA_1, LPA_2, and LPA_3 (21, 22). These LPA receptors (LPARs) were originally classified as “orphan” receptors until they were discovered to bind LPA (20). LPARs induce a variety of intracellular signaling events.

LPA receptors couple to several types of G-proteins. $G_{\alpha i}$-coupled receptors inhibit adenylyl cyclase (AC), and induce the Erk-MAPK cascade leading to activation of transcription factors, DNA synthesis and proliferation (21, 25). LPA-induced activations of Erk and Ras can usually be blocked with pertussis toxin (PTX), which indicates involvement of GPCRs (87). PTX ADP-ribosylates several G_α subunits, including $G_{\alpha i}$, thus resulting in inhibition of $G_{\alpha i}$-mediated cellular responses. $G_{\alpha q}$-coupled receptors that bind LPA activate

phospholipase C (PLC), thus resulting in Ca^{2+} mobilization and protein kinase C (PKC) activation (152). $G_{\alpha q}$ is not a target of PTX. LPA also activates intracellular phosphorylation of Akt/PKB via phosphoinositide-3 kinase (PI3K) (12, 110, 112). PI3K activation of Akt leads to activation of transcription and inhibition of apoptosis through phosphorylation of pro-apoptotic factors (19, 26). The final type of G protein-coupled LPAR is $G_{\alpha 12/13}$-coupled. This type of receptor activates Rho, which leads to stress fiber formation and cytoskeletal rearrangement (5, 92, 107).

Each LPAR can associate with more than one type of Gα subunit, although GPCRs do demonstrate preferences for particular G-proteins. In studies using knockout mice and receptor antagonists, LPA_1 has been shown to modulate tumor cell motility, suggesting coupling to $G_{\alpha 12/13}$ (50, 133). LPA_2 is associated with growth, proliferation, and tumor formation (68, 149). This role for LPA_2 suggests involvement of $G_{\alpha i}$ (149). LPA_3, through overexpression studies, has been proposed to be responsible for increases in intracellular Ca^{2+}, indicating the involvement of $G_{\alpha q}$ subunits (10). Though over-expression and knockout studies have been performed with each receptor, there appears to be some promiscuity among G-protein subunits and LPARs. For example, some studies have shown that all three LPARs can activate $G_{\alpha 12/13}$ (49). While much is known about the LPARs, the precise role of each receptor has not yet been determined.

The numerous downstream effects initiated by LPARS cause LPA to behave as a mitogen in many cell types. As part of its growth factor-like

qualities, LPA has been shown to transactivate receptor tyrosine kinases, including the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGF) (104, 111). Transactivation of growth factor receptors initiates additional intracellular signaling, in addition to the signaling initiated directly by LPARs. The topic will be addressed in more detail below.

1.3 LYSOPHOSPHATIDIC ACID IN DISEASE

Since the initial finding that LPA can be released from activated platelets (33), it has been determined that LPA is generated by a variety of mammalian cells. LPA can be released from numerous cell types including adipocytes (45), prostate cancer cells (122, 139), and ovarian cancer cells (31, 80). Accordingly, LPA acts as a mitogen factor for many cell types including pre-adipocytes (45), and corneal epithelial cells (151), as well as colon (115), ovarian (37, 79), and prostate (25) cancer cells.

The activity and generation of LPA is potentially important in many tissues and disease states. Generation of LPA results in the differentiation of pre-adipocytes to adipocytes (38). This suggests a role for LPA in development of adipose tissue and obesity (38, 98). LPA can also be produced by, and exert effects on, immune cells. LPA has been shown to activate T-cells by increasing intacellular Ca^{2+} and increasing expression of IL-2 (143). LPA can also stimulate proliferation of T-cells (47, 143) and recruit macrophages for wound healing (143).

LPA also plays a role in cancer development and progression. LPA is found in plasma at relatively low concentrations, but is present at significantly higher concentrations in ascites fluid from ovarian cancer patients (15, 144). LPA is thought to play an important role in the metastatic processes involved with cancer progression (37, 86, 120). It has been demonstrated that LPA increases cellular proliferation in various carcinoma cell lines, including ovarian and prostate cancer cells (37, 139).

LPA levels are elevated in the plasma and ascites fluid of women with ovarian cancer, as early as stage 1 (144). Ovarian cancer occurs in one out of every twenty-two women, making it a significant issue in women's health. It is the leading cause of death from gynecologic malignancy. Diagnosis and treatment of ovarian cancer are currently accomplished by invasive methods. Efforts are currently being made to discover non-invasive biomarkers so that the disease can be diagnosed and treated before progression and/or metastasis occurs. One such biomarker may be elevated levels of lysophosphatidic acid. LPARs, as well as enzymes involved in the production of LPA, may be targets for cancer therapeutics.

1.4 RECEPTOR TYROSINE KINASE TRANSACITVATION

Transactivation refers to the ability of one receptor to cause activation of another receptor via cellular signaling. One common type of receptor transactivation occurs when ligand binding to GPCRs results in subsequent

activation of growth factor receptors. LPA has been shown to activate both the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR).

Receptor transactivation occurs in numerous cell types including breast (54), ovarian (85), colon (91), and prostate cancer cells (104), as well as in the lacrimal gland (18) and in smooth muscle cells (71). This transactivation induces intracellular signaling events that lead to increases in cellular growth, proliferation, and migration in cancer cells (39, 40). Some studies have shown that some of the intracellular signaling mediated by GPCR activation can be ablated by inhibition of growth factor receptor transactivation (63). Whether the activity of the transactivated receptor accounts for all GPCR-induced mitogenic signaling has not been fully determined.

Transactivation of the EGFR in prostate cancer cells has been well documented (81, 104). This response has been shown to be induced by LPA, which induces cellular signaling events that cause a rapid increase in EGFR phosphorylation (75). This pathway has also been shown to occur in other types of carcinoma cells, including colon (91), squamous cell carcinomas (48), and ovarian cancers (85). LPA-induced EGFR transactivation can occur through several signaling pathways. These pathways are discussed in detail in the next section.

1.5 LYSOPHOSPHATIDIC ACID AND EPIDERMAL GROWTH FACTOR

EGF is a polypeptide growth factor that binds to the EGF receptor (EGFR), a transmembrane protein tyrosine kinase. EGF plays an important role in stimulating proliferation of ovarian cancer cells and other types of carcinoma cells (145). EGFRs are over-expressed by some ovarian cancer cell lines (17). EGF, along with other members of the EGFR ligand family, binds the epidermal growth factor receptor (EGFR), a member of the Her2/Neu family of receptor tyrosine kinases. This family consists of four membrane-bound proteins: EGFR, Her2, Her3 and Her4 (74, 100, 130, 147). EGF and other EGFR ligands, which include transforming growth factor α (TGFα), heparin binding EGF (HB-EGF), and amphiregulin, bind to and activate the EGFR. Binding of EGF to its receptor initiates receptor dimerization and autophosphorylation. The autophosphorylation of the EGFR initiates various intracellular signaling pathways that lead to growth and proliferation of cells (6). Growth stimulation by EGF is linked to the activation of Ras-MAPK pathway. The PI3K pathway can also be activated by heterodimers involving Her3, the only EGFR to bind directly to the 85kd subunit of PI3K (66, 121).

It has been shown that LPA can stimulate transactivation of the EGF receptor in some cell types (90, 104). There are at least two ways in which this cross-talk can occur. First, LPA activates matrix metalloproteases (MMPs) that can cleave membrane pro-HB-EGF to HB-EGF (81), which can then bind to and activate the EGFR (48, 81, 104). HB-EGF is a member of the EGF family of

ligands that binds EGFR subunits, EGFR and Her4 (34, 55). This pathway is depicted in Figure 1-1. Second, intracellular transactivation can occur via phosphorylation of the EGFR by non-receptor tyrosine kinases, such as c-Src. LPA does activate c-Src, and it has been hypothesized that c-Src can transactivate the EGFR intracellularly (2, 15, 16).

Conversely, some growth factors can activate LPA production, potentially resulting in transactivation of LPA receptors (139). Our lab has shown that LPA production, stimulated by the peptide bombesin, can lead to LPAR activation (139). Many growth factors, including EGF, can stimulate phospholipase D (PLD) (59, 118), an enzyme that may be involved in LPA generation. This dissertation project further explores pathways for agonist-induced LPA production, with particular emphasis on the role of PLD2.

1.6 LYSOPHOSPHATIDIC ACID METABOLISM PATHWAYS

LPA can potentially be generated through several enzymatic pathways. Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA), which then can potentially be converted to LPA by phospholipase A_1 or A_2 ($PLA_{1/2}$). Phosphatidylcholine-utilizing PLD exists in two isoforms, PLD1 and PLD2, which are widely expressed and are about 50% identical in amino acid sequence (46).

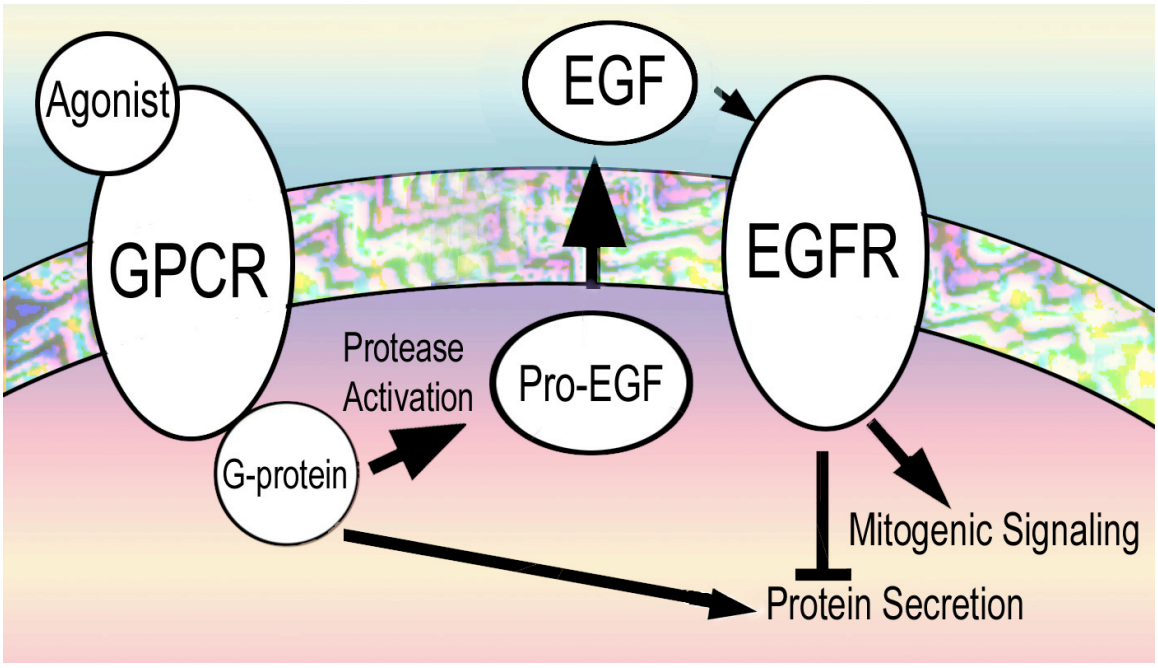


Figure 1-1. Transactivation of the EGFR by GPCRs (61). Transactivation of the EGFR by GPCR agonists involves activation of proteases such as MMPS. These proteases cleave pro-forms of EGF from the membrane, which then bind to and activate the EGFR. This transactivation results in mitogenic signaling and, in some cell lines (61), in decreased protein secretion.

We have previously shown that various cell lines express one or both forms of PLD (46). Cellular PLD activity can be stimulated by growth factors, such as EGF, as well as by agonists binding to G-protein coupled receptors (36). Mammalian PLD1 (120kDa) can be activated via PKC isoforms, phosphatidylinositol bisphosphate (PIP2), ARF, and other small GTPases (93). PLD2 (105kDa) is also agonist activated and stimulated by PIP2, but its mode of regulation is less clear. PLD2 has been shown to directly interact with phospholipase Cγ (PLCγ) following EGF stimulation (30, 59). PLD2 has also been shown to form a complex with the EGF receptor (118).

Another pathway for LPA production involves lyso-PLD, which converts lyso-PC to LPA (129, 140). Relatively little is known about the activation and regulation of lyso-PLDs. Some forms of lyso-PLD circulate in plasma (45). Plasma lyso-PLD activity has recently shown to be conferred by autotaxin (ATX).

Autotaxin is an ectoenzyme that is released from a plasma membrane precursor. Upon release from the membrane, ATX converts lyso-PC to LPA (131). Autotaxin was first discovered as a motogen produced by human melanoma cells (105, 124). Since this finding, ATX has been shown to induce LPA production, proliferation, and motility in a variety of cell lines (50, 70, 131). It is unlikely that this enzyme accounts for all lyso-PLD activities or LPA production, since other forms of lyso-PLD have been characterized at the activity level (141). ATX activity has not been shown to be subject to agonist regulation.

Acylglycerol kinase (AGK) is yet another enzyme that can produce LPA. AGK, which is found in mitochondria, phosphorylates monoacylglycerol to form

LPA (13). Although regulation of AGK has not been directly demonstrated, EGF-stimulated Erk activation is attenuated when AGK levels are decreased using siRNA. This result potentially implicates AGK in EGFR-mediated signaling (13, 122).

LPA is dephosphorylated and inactivated by lipid phosphate phosphatases (LPPs) (52, 69). This dephosphorylation event attenuates the activities of LPA (126), hydrolyzing LPA to monacylgycerol (MG) and an inorganic phosphate group. Agonist regulation of LPP activities has not been described. However, these enzymes have been shown to be less active in ovarian cancer patients as well as within ovarian cancer cell lines, thus increasing LPA levels as compared to control patients or cells (126). When LPPs are over-expressed in ovarian cancer cell lines, they decrease the response to exogenous LPA (127). These findings indicate that LPPs may play an important role in LPA accumulation in ovarian carcinomas.

LPA can also be inactivated by lysophophosphatidic acid acyl transferases (LPAATs), which convert LPA back to PA (78). LPAAT exists in two isoforms, LPAAT-α and LPAAT-β. While LPAAT-α is expressed in all tissues, LPAAT-β is differentially expressed, with the highest levels found in the heart and liver (78). LPAATs could potentially play a role in downregulating LPA-mediated responses.

1.7 PHOSPHOLIPASE D2

PLD2 plays an important role in adhesion and in the regulation of intracellular signaling, as well as in lipid metabolism. PLD2 contains a pleckstrin homology domain (PH), which has been shown to be important in protein-protein interactions (101). The PH domain binds phosphatidylinositol bisphosphate (PIP2) (3), which is needed for PLD2 activation (28). The relatively high basal activity of PLD2 is attributed to basal levels of PIP2 in cells (102). PLD2 also contains two PLD catalytic domains, termed HKD domains. Upon mutation of either of these domains, PLD2 activity is lost (36).

The cellular roles of PLD2 remain to be fully elucidated. However, it is clear that PLD2 can influence cell adhesion events. It has been suggested that PLD2 can stimulate the initial stages of adhesion through β_1 and β_2 integrins, via generation of phosphatidyl alcohols (PtdOHs) (102). In contrast, PLD2 has also been reported to negatively regulate focal adhesion kinase (FAK) and paxillin in PC12 prostate cancer cells (7). Work in our lab has shown that the major phenotypic effect of PLD2 over-expression in lymphoma cells is enhanced adhesion (Knoepp et. al., in preparation).

Although the mechanisms regulating PLD activity are still being elucidated, it is clear that PLD2 can be regulated by agonists (46). EGF can stimulate the activity of both PLD1 and PLD2 in HEK293 cells (118). PLD2, but not PLD1, forms a physical complex with the EGFR in HEK293 cells, and becomes tyrosine phosphorylated upon EGF stimulation (118). This response

has also been seen in other cell lines, including A431 epidermoid carcinoma cells (83). Since tyrosine phosphorylation has not been shown to regulate the activity of PLD2, the relevance of this occurrence is unknown.

1.8 LYSOPHOSPHATIDIC ACID RECEPTOR ANTAGONISTS

In recent years much effort has been directed toward the development of LPAR antagonists. Two such compounds have proven to be experimentally useful in delineating LPAR-mediated responses. Ki16425 has proven to attenuate many LPA induced responses. Ki16425 can inhibit responses mediated by all three LPARs, in the rank order $LPA_1 \geq LPA_3 >> LPA_2$ (97). Ki16425 blocks LPAR-mediated signaling events such as DNA synthesis and cellular migration (97, 151). Ki16425 has also been shown to attenuate ascites-induced migration of pancreatic cancer cells (146). VPC 32179, another LPA1/3 antagonist, also attenuates ascites-induced migration of pancreatic cancer cells (146), and blocks the proliferation of mast cells (8). Inhibition of LPARs may prove to be a viable therapeutic approach for some types of cancers.

1.9 OVARIAN CARCINOMA BIOLOGY

Ovarian cancer is the most lethal of all gynecological malignancies, due primarily to its detection in late stages of the disease (106). Ovarian cancer development is classified into stages 1-4. The stages are based on tumor size,

involvement of the lymph nodes, and determination of metastases. Stage 1 ovarian cancer involves one or both ovaries; stage 2 involves both ovaries with pelvic extension (99). In stage 3 the tumor involves both ovaries with metastasis confined to the peritoneal cavity and/or the lymph nodes (99). The final stage, stage 4, involves all of the previously described sites as well as distant metastasis (99). The cure rate for ovarian cancer detected in stage 1 is 90% (11). However, only 25% of ovarian cancers are detected in stage 1; the cure rate drops to only 20% in cases diagnosed at a more advanced stage (11). Despite developments in chemotherapeutic approaches, little change has been observed in the mortality rate of ovarian cancer over the last 20 years (32).

The most important risk factor for development of ovarian cancer, other than advanced age, is family history (35, 94). This can be attributed in some cases to the inheritance of mutated breast and ovarian cancer susceptibility genes 1 and/or 2 (BRCA1/2) (76, 94). Mutations in these two genes currently serve as genetic markers for women at risk for both breast and ovarian cancer (82). Interestingly, oral contraceptive use, a risk factor for breast cancer, is suggested to decrease the risk of development of ovarian cancer by 30% - 60% (1).

Primary treatment of ovarian cancer is determined by the stage of the disease (99). Surgery to remove the affected ovary or ovaries is the first line of defense against spread of the disease, or against its further spread if the patient is already in an advanced stage (99). Chemotherapeutic agents are also used in conjunction with surgery to ablate the entire tumor mass, and to prevent

metastasis. The three most common chemotherapeutics currently used for treatment of ovarian cancer are paclitaxel (Taxol) and carboplatin or cisplatin.

LPA appears to play an important role in the development and progression of ovarian cancer. LPA levels are elevated in the acites fluid surrounding ovarian tumors (137), as well as in the plasma of ovarian cancer patients (144). These findings could implicate LPA as a biomarker for early detection of ovarian cancer. LPA increases the invasiveness and metastatic potential of ovarian cancer cells by increasing matrix metalloprotease activity, allowing tumor cells to invade surrounding tissues and blood vessels (119). Vascular endothelial growth factor (VEGF) expression is also increased by LPA, thus aiding in tumor angiogenesis (42, 57). LPA can also mimic EGFR signaling by increasing amounts of free HB-EGF in the extracellular space (136). Thus, strategies interfering with LPA production and/or response maybe of therapeutic benefit in ovarian cancer.

1.10 HYPOTHESIS AND PROJECT OVERVIEW

The major hypothesis addressed in this dissertation is that epidermal growth factor stimulates lysophoshphatidic acid production through activation of cellular phospholipase D2. At the time with this project was initiated, it was known that LPA production could be stimulated by agonists, but the enzymatic pathways involved had not been established. Our original focus was on the potential role of PLD2, which our lab has shown to be an agonist-activated enzyme. As the project progressed, information became available concerning new enzymes (e.g., autotaxin, acylglycerol kinase) that might be involved. Thus, this project examines a variety of enzymes and signaling pathways.

PROJECT OVERVIEW

Aim I. To characterize EGF-stimulated LPA production in ovarian cancer cell lines.

Preliminary data indicated that EGF simulates LPA production in the media of two ovarian cancer cell lines, OVCAR-3 and SKOV3. We determined the dose-response and time course of EGF-induced LPA production in these cells. Radiolabeling and mass spectrometry approaches were used to detect LPA. Finally, we tested the role of potential cross-talk between EGF and LPA receptors in LPA production by using receptor inhibitors and antagonists.

Aim II. To determine the role of selected enzymes in EGF-induced LPA production.

We used isotopic and fluorescent assays to characterize intrinsic PLD and lyso-PLD activities of ovarian cancer cell lines. We determined which enzymes were stimulated by EGF. We used over-expression and siRNA approaches to increase or decrease levels of PLD2 and ATX, two enzymes potentially involved in LPA production. The effects of these manipulations on LPA production were then assessed.

CHAPTER II

EFFECTS OF EPIDERMAL GROWTH FACTOR ON LYSOPHOSPHATIDIC ACID PRODUCTION IN OVARIAN CARCINOMA CELLS

2.1 SUMMARY

Lysophosphatidic acid (LPA), a lipid mediator involved in diverse cellular processes, binds to the Edg family of G-protein coupled receptors to induce a myriad of cellular responses in tumor cells. Epidermal growth factor (EGF), a polypeptide growth factor, binds to the EGF receptor (EGFR), a receptor tyrosine kinase. Like LPA, EGF induces various cellular responses in tumor cells including proliferation, induction of angiogenesis, migration, and metastasis. LPA has the potential to act as an autocrine/paracrine factor. This study explores cross-talk between the EGF and LPA receptors. We found that EGF and LPA both cause tyrosine phosphorylation of the EGFR in two human ovarian cancer cell lines, OVCAR3 and SKOV3. Both agonists also activate the downstream signaling kinases, Erk and Akt. Of particular interest, both EGF and LPA stimulate LPA production by OVCAR3 and SKOV3 cells. PD158780, an EGFR-selective tyrosine kinase inhibitor, blocks LPA production in response to EGF and LPA in both cancer cell lines. Ki16425, an LPA1/LPA3 receptor antagonist, inhibits EGF- and LPA-induced LPA production in SKOV3 cells. These results indicate that binding of EGF to its receptor stimulates LPA production, and suggest that cross-talk can occur bidirectionally between EGF and LPA receptors.

2.2 INTRODUCTION

Lysophosphatidic acid (LPA) is a biologically active lipid mediator. LPA has been shown to induce proliferation, migration, and metastasis of tumor cells (14, 79). LPA binds to G-protein coupled receptors LPA_1, LPA_2, and LPA_3 to induce these effects (22), which are mediated through both the Erk MAPK and Akt pathways (12, 79, 110). LPA receptors (LPARs) are widely in expressed in many tissues, including the ovary (135), but the exact roles of each receptor subtype are still being explored (149).

LPA is found in plasma at relatively low concentrations, but is present at higher concentrations in ascites fluid from ovarian cancer patients (144). LPA is thought to play an important role in the metastatic processes involved with cancer progression. It has been demonstrated that LPA increases proliferation in various carcinoma cell lines, including prostate cancer cells (31, 139). In some cells, this process involves ErkMAPK activation via a Ras-dependent pathway (25). LPA-induced proliferation can also be dependent on the PI3K/Akt pathway (151). LPA-induced activation of Erk and Ras can in many cases be blocked by pertussis toxin, indicating involvement of G-protein coupled receptors (87).

Epidermal growth factor (EGF), a polypeptide growth factor, binds to the EGF receptor, a transmembrane protein tyrosine kinase. EGF stimulates proliferation of ovarian cancer cells and other types of carcinoma cells (17). Growth stimulation by EGF is mediated in part by the Ras/Erk pathway, as is the

case for LPA. The PI3K/Akt pathway also plays a prominent role in EGF responses (60).

It has been shown that LPA can stimulate transactivation of the EGF receptor in some cell types (89, 90, 136). LPA can activate matrix metalloproteases (MMPs), which cleave pro-EGF forms from the membrane (104). Intracellular signaling proteins, such as PLC and PLD, have also been suggested as mediators of LPA-induced receptor tyrosine kinase transactivation (24, 62). Conversely, some growth factors and cytokines can activate LPA production, resulting in transactivation of the LPA receptors (139, 151).

EGF and LPA have been shown to induce common signaling pathways such as Erk and Akt, which lead to growth and proliferation of tumor cells (17, 110, 113). Thus, these two agonists activate similar downstream signaling events, and some data suggest that LPA-induced mitogenic signaling is mediated primarily through EGFR transactivation (48, 75). However, it is still being determined if EGFR transactivation accounts for all LPA-mediated mitogenic signaling events.

In this study we used OVCAR3 and SKOV3 adenocarcinoma cell lines as models to examine the cross-talk between EGF and LPA. The results suggest that LPA can act as an autocrine mediator, downstream of EGFR.

2.3 MATERIALS AND METHODS

Materials: LPA (oleoyl 18:1) was from Avanti Polar Lipids, Inc. (Birmingham, AL). [^{3}H]-Palmitic acid was from Amersham Biosciences (Piscataway, NJ) or from Perkin Elmer (Wellesley, MA). Anti-phospho-EGFR (Tyr 992), anti-phospho-Akt (Ser473), anti-phospho-44/42 MAPK (Thr202/Try204) and anti-p44/42 MAPK were from Cell Signaling. Technologies (Beverly, MA). PD158780 and PTX were obtained from Calbiochem (San Diego, CA). Ki16425 and EGF were from Sigma (St. Louis, MO).

Cell culture: SKOV3 and OVCAR3 cells obtained from the American Type Culture Collection (Manassas, VA) were maintained in RMPI 1640 medium (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), and McCoy's 5A (Cellgro, Herndon, VA) medium supplemented with 10% fetal bovine serum, respectively. All cells were grown at 37°C in 5% CO_2/ 95% air on standard tissue culture plastic. Cells were serum-starved by incubation in serum-free medium for 12-24 hours prior to experiments.

Immunoblotting: Whole-cell extracts were prepared from cells using a lysis buffer containing 1M HEPES, 1M NaCl, 0.1M EGTA, 1M β-glycerophosphate, 0.2M Na-pyrophosphate, 0.01M Na-ortho vanadate, 1% Triton X-100, 0.1M PMSF, 10mg/ml aprotinin, and 10mg/ml leupeptin (46). Protein concentrations were determined using Coomassie blue reagent (Pierce Chemical Co., Rockford, IL).

Equal amounts of protein were loaded (100μg) on each lane of a Laemmli 10% SDS polyacrylamide gel. Proteins were separated by PAGE and transferred to PVDF. Blots were developed using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Isotopic method for LPA analysis: LPA production in cells and medium was assessed as described previously (140). Briefly, cells were grown in 6-well plates and metabolically labeled with 5μCi/dish [^{3}H]-palmitic acid in serum-free medium for 12-24 hr. Prior to treatment, the labeled cells were incubated in Dulbecco's modified Eagle's medium supplemented with 10mM HEPES (pH 7.5) at 37°C for one hour. Agonists and/or antagonists were then added for varying periods of time. Following treatment, methanol/HCl and chloroform were used to extract lipids from cells and medium, as previously described (140). Lipids were dried under nitrogen and resuspended in chloroform/methanol. Oleoyl (18:1)-LPA was added to each tube as a standard. Thin-layer chromatography, on an oxalic acid-impregnated silica gel plate, was used to separate the lipids. The LPA standard was visualized by iodine staining. [^{3}H]-labeled lipids were localized by using autoradiography after spraying the plates with Enhance (PerkinElmer Life Sciences. Inc., Boston, MA). The LPA band, as well as the other lipids in the lane, were separately scraped from the TLC plate and quantified using liquid scintillation spectrometry.

Mass spectrometry analysis of LPA: Cells were grown in 6-well tissue culture plates and serum-starved 12 hours prior to experiments. Following serum-starvation cells were washed with Dulbecco's modified Eagle's medium supplemented with 10mM HEPES (pH 7.5) at 37°C for one hour. Methanol/HCl and chloroform were used to extract lipids from cells and medium, as previously described (140). Lipids were dried under nitrogen and resuspended in chloroform/methanol. An aliquot of 17:0 (non-endogenous) LPA was added to each tube as a standard. Lipids were separated by thin-layer chromatography on an oxalic acid-impregnated silica gel plate. The LPA standard was visualized by iodine staining. LPA was extracted from the silica gel by sequential addition of chloroform, methanol, and water (20 minutes each). Samples were then filtered with a 0.22μm hydrophilic filter under vacuum and direct syringe injected into a Thermo Finnigan TSQ 7000 Mass Spectrometer and run in negative ion mode.

Statistical Analysis: All experiments were repeated; representative results are shown. Statistical significance was determined using one-way ANOVAs in Graph-Pad InStat.

2.4 RESULTS

Effects of EGF and LPA on ovarian cancer cell signaling

LPA has been shown to transactivate the EGF receptor in other cell types, but this pathway had not previously been assessed in ovarian cancer cells. We used immunoblotting with a phospho-EGFR (Tyr 992) antibody to assess phosphorylation/activation of the EGFR in a time course experiment using both EGF and LPA as agonists. OVCAR3 and SKOV3 cells were stimulated with 10nM EGF or 10μM 18:1 LPA for time points from 5-60 minutes. As shown in Figure 2-1, phosphorylation of the EGFR increased in response to EGF. LPA induces a similar phosphorylation of the EGFR. In OVCAR3 cells, EGF induced rapid EGFR phosphorylation that declined after 30 minutes of stimulation. LPA induced a more sustained EGF phosphorylation in OVCAR3 cells, with stimulation still occurring at 60 minutes. In SKOV3 cells, EGFR phosphorylation was visible after 5 minutes of stimulation with both EGF and LPA, and remained elevated at 60 minutes. Thus, although the kinetics of EGFR varied between the two cell lines, LPA-induced transactivation of the EGFR was apparent in both OVCAR3 and SKOV3 cells.

The effects of EGF and LPA on intracellular signaling cascades were examined. In OVCAR3 cells, EGF activated Erk1/2 to a greater extent than did LPA, although the response to LPA was more prolonged. LPA induced more pronounced Akt phosphorylation than did EGF; Akt activation was sustained for

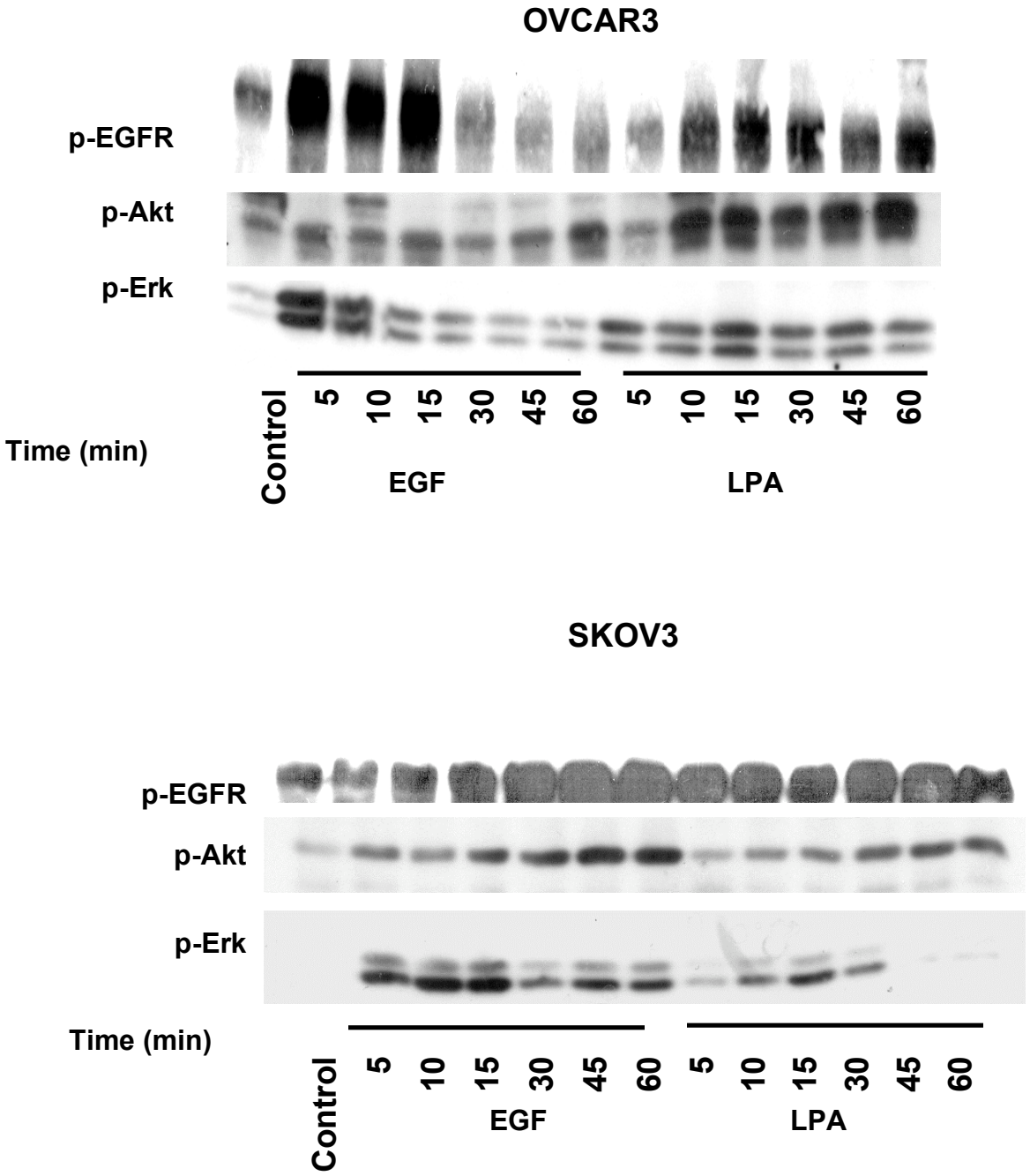


Figure 2-1. Effects of EGF and LPA on intracellular signaling in ovarian cancer cells. OVCAR3 and SKOV3 cells were serum-starved for 12 hours and then treated with 10nM EGF or 10μM 18:1 LPA for 5 to 60 minutes. Whole cell lysates (100μg protein) were loaded and separated on a 10% SDS-PAGE gel. Membranes were immunoblotted for phospho-EGFR (Tyr 992), phospho-Erk1/2, and phospho-Akt.

60 minutes. In SKOV3 cells, EGF induced Erk and Akt phosphorylation to a greater extent than did LPA. Again, Akt phosphorylation was sustained longer than Erk phosphorylation, for both agonists. Taken together, these data suggest the potential for EGFR transactivation as a pathway for some LPA-mediated responses. However, since LPA-induced Akt activation precedes EGFR transactivation in OVCAR3 cells, it is clear that LPA can induce responses independently of EGF.

Effects of EGF on LPA Production

Given that LPA can transactivate the EGFR in ovarian cancer cells, and that many of the same intracellular signaling pathways are activated by EGF and LPA, we next examined the effects of EGF on LPA production. Using a metabolic labeling assay (140), we analyzed LPA production in both OVCAR3 and SKOV3 cells (Figure 2-3). Results obtained previously in our lab, using other cell lines, indicated that agonist-induced LPA production is most prominent in cell culture medium (4). We examined effects of 10nM EGF on LPA levels from 5 -120 minutes. Lipids from the media and cells were harvested separately. LPA levels increased in the medium as early as 15-30 minutes and plateaued at 60-120 minutes (Figure 2-3a). In the same experiment, LPA levels within OVCAR3 cells were elevated by ~40%. EGF did not detectably increase LPA levels within SKOV3 cells (Figure 2-3b).

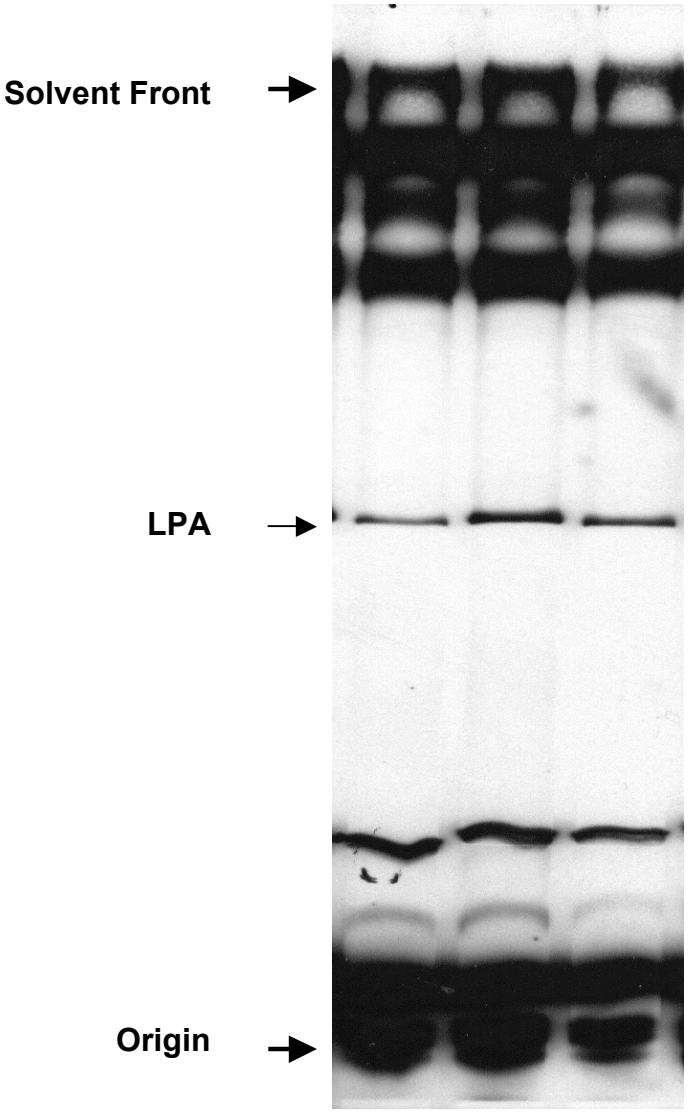
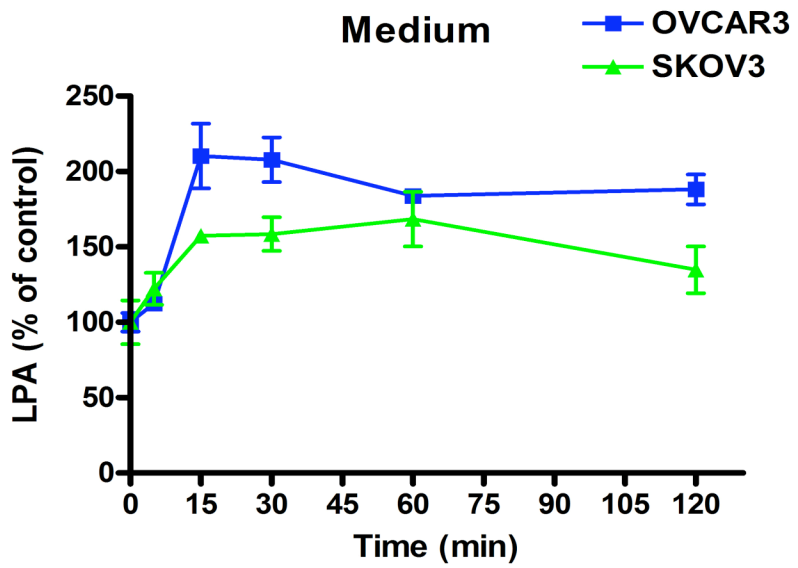


Figure 2-2. Representative film from a TLC plate showing separation of LPA. OVCAR3 were metabolically labeled with [^{3}H]-palmitic acid in serum-free medium for 12 hours and then washed with DMEM. Lipids were harvested from the medium and separated by TLC. Each sample was spiked with 18:1 LPA as a standard and visualized with iodine. The plate was subjected to autoradiography, as shown. The lanes shown represent results from triplicate dishes of cells.

A



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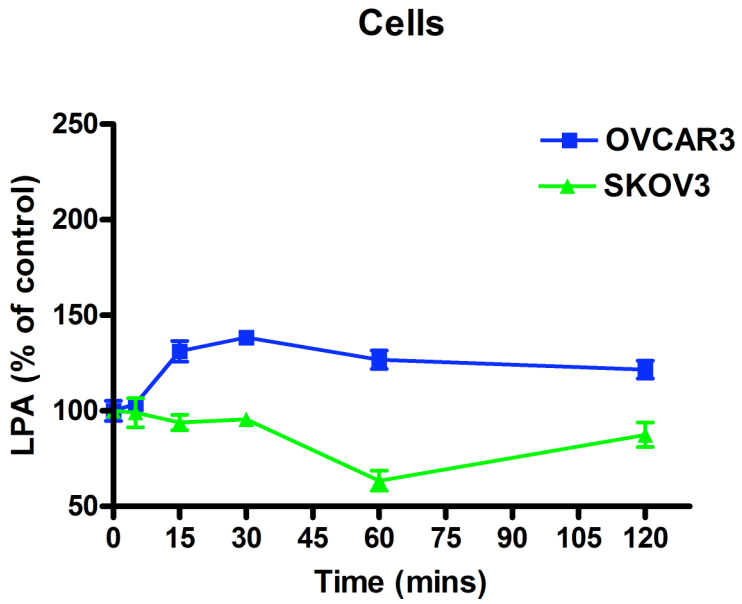

Figure 2-3. Time course for LPA production by ovarian cancer cell lines. OVCAR3 and SKOV3 cells were metabolically labeled with [^{3}H]-palmitic acid and then stimulated with 10nM EGF for time from 5 to 120 minutes. Medium (A) and cells (B) were harvested separately; lipids were separated using TLC. Liquid scintillation counting was used to quantify LPA and other total lipids. LPA is represented as a percent of the total lipid recovered from cells or medium. The values expressed are normalized to the untreated control and indicate mean ± SEM for triplicate dishes of cells.

Mass spectrometry analysis of LPA in ovarian cancer cells

We performed mass spectrometry to confirm the presence of LPA in the samples analyzed for LPA production. Medium was harvested from OVCAR3 cells and lipids were separated as described for the isotopic LPA assay. The LPA band, identified by addition of 17:0 (non-endogenous) LPA, was harvested from the TLC plate. LPA was extracted and then directly syringe injected into the mass spectrometer. Results were analyzed in negative ion mode. As shown in Figure 2-4, the 17:0 standard was detected as well as cellular 18:1 LPA. These results confirm that endogenous LPA is present, as expected, in the samples analyzed for LPA production assays.

Effects of EGFR inhibition on LPA production

EGF-induced LPA production is presumably mediated through EGFR-mediated activation of phospholipases. In order to test whether increased LPA production was dependent on EGFR catalytic activity, PD158780 (an EGFR-specific tyrosine kinase inhibitor) (123) was used to block EGFR activation. OVCAR3 cells were incubated with 10pM PD158780 for 5 minutes, then stimulated with 10nM EGF for 5 minutes. PD 150780 blocked phosphorylation of the EGFR in OVCAR3 cells (data not shown), as expected.

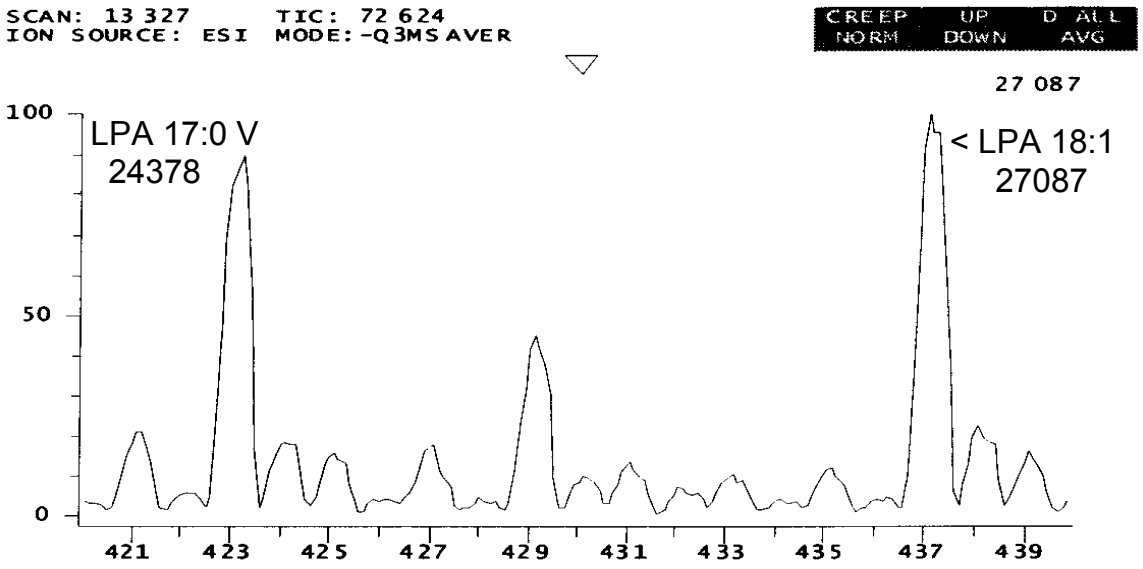

Figure 2-4. Mass spectrometry analysis of LPA from OVCAR3 medium. OVCAR3 cells were serum-starved for 12 hours and then washed in DMEM. Medium was harvested and lipids separated by TLC using 17:0 (non-endogenous) LPA as a standard. LPA was extracted from the silica gel by sequential addition of solvents. Samples were then filtered and direct syringe injected into the Thermo Finnigan TSQ7000 mass spectrometer. The mass spectrum shown is a representative spectrum from untreated control cells. Peaks for 17:0 and 18:1 LPA are indicated.

Next, the effects of EGFR inhibition on LPA production were tested. OVCAR3 and SKOV3 cells were treated with 10pM PD158780 for 5 minutes. The cells were then incubated with 10nM EGF or 10 μM 18:1 LPA for 30 minutes, and LPA production was measured. PD158780 blocked the EGF-induced increases in LPA levels in both cell lines (Figure 2-5A). Interestingly, the inhibitor also interfered with LPA-induced LPA production (Figure 2-5B). These data suggest that EGFR activation, either directly through ligand binding or indirectly though transactivation respectively, is necessary for EGF-and LPA-induced LPA production.

Effects of LPAR inhibition on LPA production

We tested the ability of an LPAR antagonist to interfere with agonist-induced LPA production. We used a recently developed LPA receptor antagonist, Ki16425, that can antagonize LPA_1, LPA_2 and LPA_3, but has higher affinity for LPA_1 and LPA_3 (97). OVCAR3 and SKOV3 cells were pretreated with 10uM Ki16425 for 5 minutes and then stimulated with 10nM EGF or 10μM 18:1 LPA for 30 minutes. The medium was harvested; lipids were separated and quantified. Agonist-induced increases in LPA levels were decreased in SKOV3 cells treated with Ki16425 (Figure 2-6A). Interestingly, responses to both EGF and LPA were inhibited. Assuming that Ki16425 has the desired specificity, this result suggests that transactivation of LPARs is critical for EGF-induced LPA production. Use of Ki16425 in OVCAR3 cells was problematic. These cells

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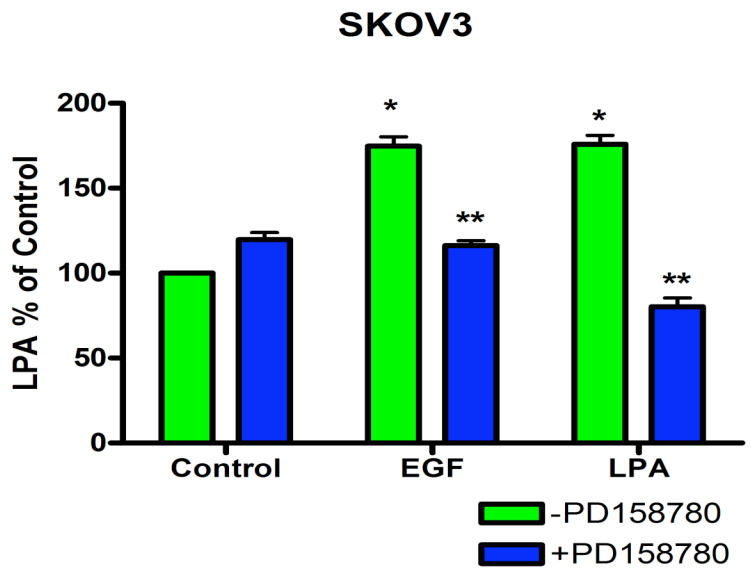

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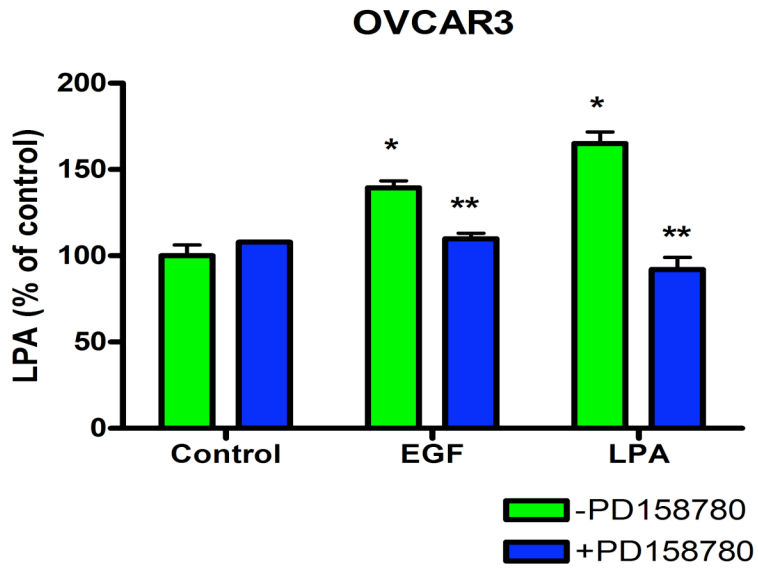

Figure 2-5. Effects of EGFR inhibition on agonist-induced LPA production. OVCAR3 and SKOV3 cells were metabolically labeled with [^{3}H]-palmitic acid and then pretreated with 10pM PD158780 for 5 minutes. Cells were then stimulated with 10nM EGF or 10μM 18:1 LPA for 30 minutes. Medium was harvested and the lipids separated by TLC. LPA was quantified using liquid scintillation counting. The data are normalized to the untreated controls and represent triplicate mean ± SEM of values from dishes of cells.(* represents agonist vs. control, ** represents treatment+PD158780 vs. treatment-PD158780, $p<0.01$).

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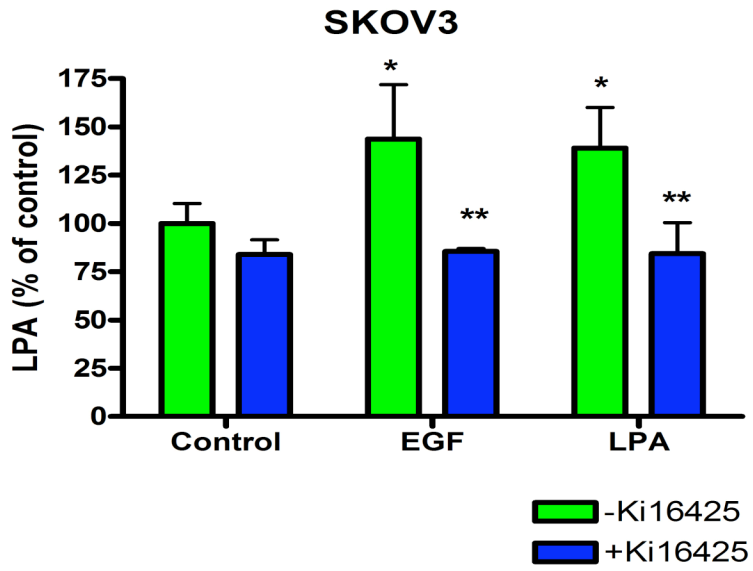

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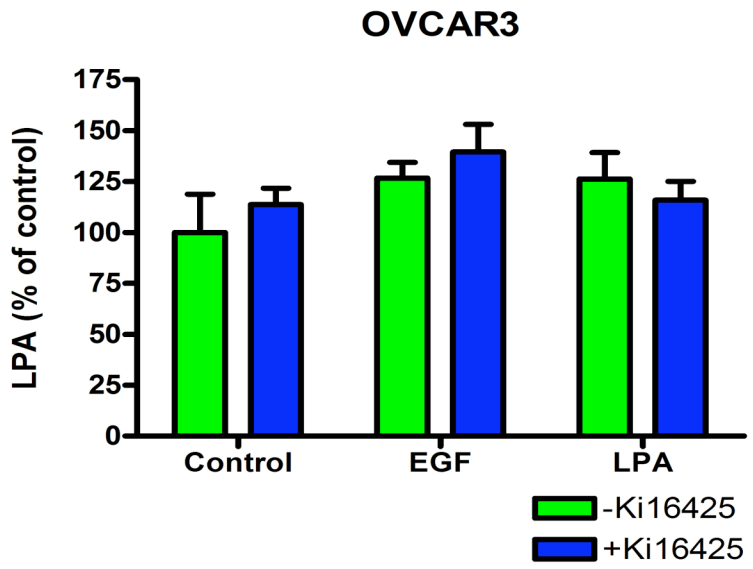

Figure 2-6. Effects of Ki16425 on LPA production in ovarian cancer cells. OVCAR3 and SKOV3 cells were metabolically labeled with [^{3}H]-palmitic acid for 12 hours and then pretreated with 10μM Ki16425 for 5 minutes prior to stimulation with 10nM EGF or 10μM 18:1 LPA for 30 minutes. Medium was harvested and the lipids separated and quantified. The values expressed are normalized to the untreated controls and represent mean ± SEM of values from triplicate dishes of cells (* represents agonist vs. control, ** represents treatment+Ki16425 vs. treatment-Ki16425, $p<0.05$).

consistently exhibited an increase in LPA production with Ki16425 (Figure 2-6B), as well as an increase in Erk phosphorylation (Figure 2-7). Ki16425 did not inhibit LPA-mediated Erk activation, but instead increased basal levels of Erk phosphorylation, indicating that Ki16425 does not always act as a pure antagonist. Since Ki16425 is an LPA analog, it conceivably could behave as a partial agonist. We also tested VPC 32183, an alternate LPA receptor antagonist, with no improvement in the results.

Pertussis Toxin (PTX), an inhibitor of $G_{\alpha i}$ and $G_{\alpha o}$, was used to determine the role of these G-proteins in EGF-and LPA-induced responses. In other cell types PTX inhibits many, but not all, responses to LPA. OVCAR3 and SKOV3 cells were incubated with 100ng/ml PTX overnight in serum-free medium. Cells were then incubated with 10nM EGF or 10μM 18:1 LPA for 30 minutes to assess LPA production. Medium was harvested, lipids were extracted, and LPA was quantified. In OVCAR cells, PTX significantly inhibited LPA-induced LPA production (Figure 2-8), as well as EGF-induced LPA production. Similar results were obtained in SKOV3 cells (Figure 2-8). Together with the EGFR inhibition data, the pertussis toxin data suggest that EGFR transactivation is mediated by $G_{\alpha i}$. Inhibition of $G_{\alpha i}$, with PTX blocks both EGF- and LPA- mediated responses by inhibiting LPA-induced transactivation of the EGFR.

The effects of PTX on other intracellular responses were also assessed. PTX did not affect EGF-or LPA-induced Erk or Akt phosphorylation (data not shown). These data suggest that some LPA responses in these two cell lines are PTX-insensitive.

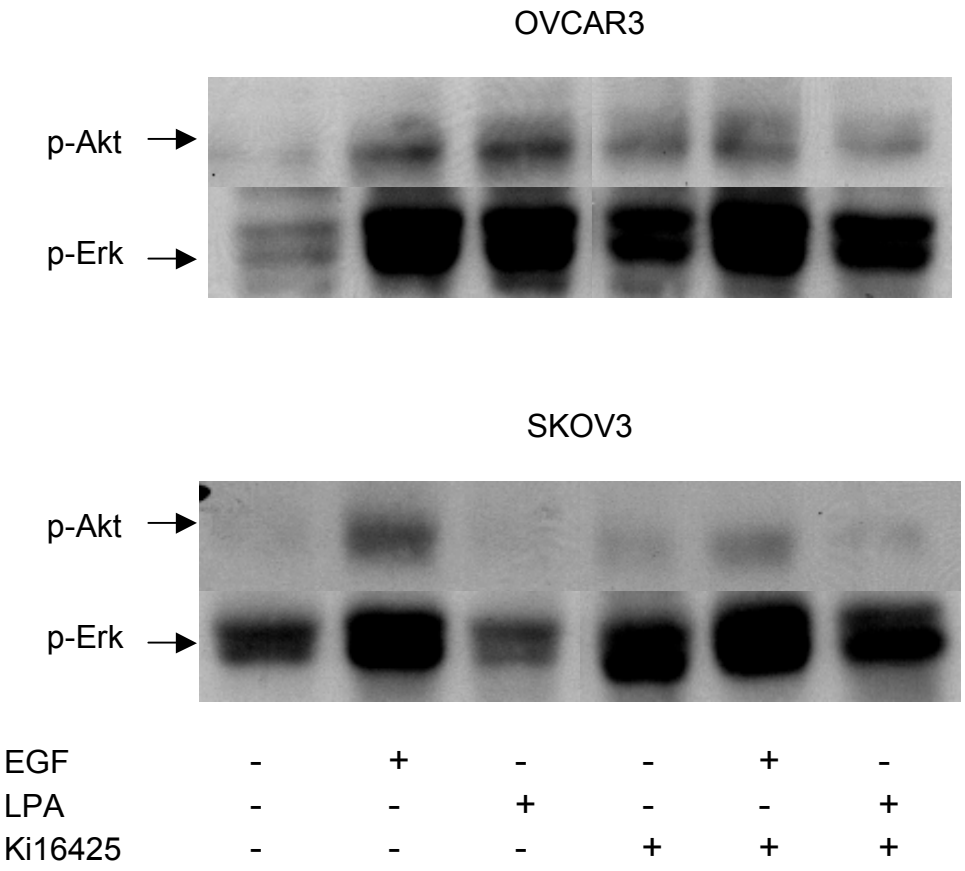

Figure 2-7. Effects of Ki16425 on intracellular signaling. OVCAR3 and SKOV3 cells were serum starved for 12 hours, pretreated with 10μM Ki16425 for 5 minutes, and then incubated with 10μM 18:1 LPA or 10nM EGF for 5 minutes. Whole cell extracts (100μg protein) were separated by SDS-PAGE and transferred to PVDF. Membranes were immunoblotted for phospho-Erk1/2 and phospho-Akt.

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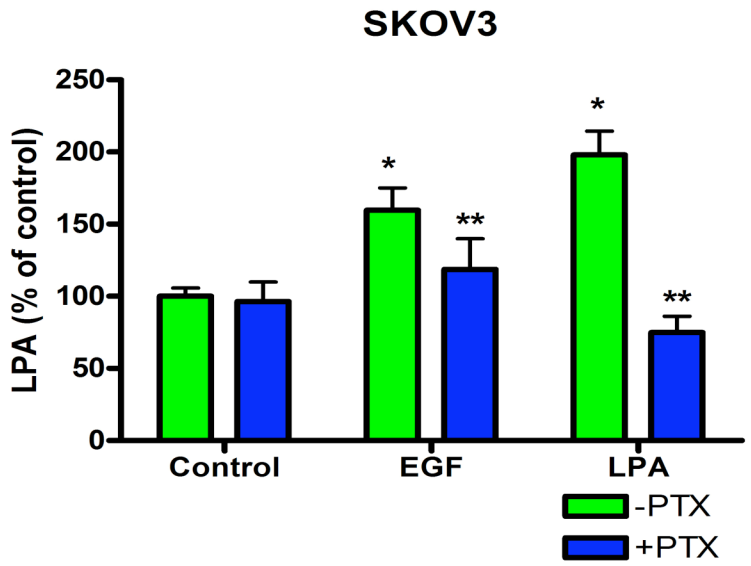

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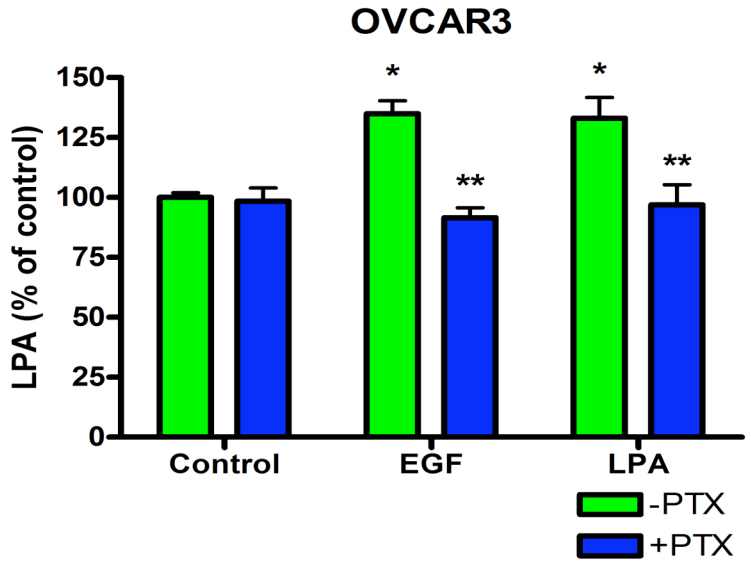


Figure 2-8. Effects of PTX on LPA production in ovarian cancer cells. SKOV3 and OVCAR3 cells were metabolically labeled with [^{3}H]-palmitic acid and preincubated with 100ng/ml PTX for 12 hours. Next, cells were incubated with 10nM EGF or 10μM 18:1 LPA for 30 minutes. Medium was harvested and the lipids separated and quantified. The values expressed are normalized to the untreated controls and represent mean ± SEM of values from triplicate dishes of cells (* represents treatment vs. control, ** represents treatment+PTX vs. treatment-PTX, $p<0.05$).

Effects of Inhibition of Intracellular Signaling Molecules

LPA and EGF both activate the Erk-MAPK pathway, which promotes proliferation of carcinoma cells (17, 72). Inhibition of this pathway has been shown to reduce EGF-and LPA-induced proliferation (151). In addition, Erks can phosphorylate and activate cPLA2 (84, 142). We asked whether inhibition of Erk with UO126, a MEK inhibitor, or inhibition of Akt with LY294002, a PI3K inhibitor, would effect LPA-or EGF-induced LPA production. First we tested whether UO126 would block Erk activation and LY294002 would block Akt phosphorylation under the conditions used. OVCAR3 and SKOV3 cells were serum starved overnight, pretreated with or without 25μM LY294002 or 10μM UO126 for 5 minutes, and then incubated with10nM EGF or 10μM 18:1 LPA for 5 minutes. UO126 blocked Erk phosphorylation in both cell lines (Figure 2-9). Interestingly, UO126 slightly enhanced Akt activation, suggesting negative cross-talk between the Erk and Akt pathways. LY294002 blocked LPA-and EGF-induced Akt phosphorylation in both OVCAR3 and SKOV3 cells.

OVCAR3 and SKOV3 cells were metabolically labeled for 12 hours, then incubated with or without 25μM LY294002 or 10μM UO126 for 5 minutes prior to treatment with 10nM EGF or 10μM 18:1 LPA for 30 minutes. The medium was harvested; lipids were separated by TLC and quantified. Addition of UO126 to OVCAR3 cells decreased EGF and LPA-induced LPA production (Figure 2-10). Use of UO126 in SKOV3 cells was problematic, in that the inhibitor significantly decreased basal LPA levels. LY294002 decreased EGF- and –LPA-induced LPA production in OVCAR3 cells, and reduced basal levels of LPA

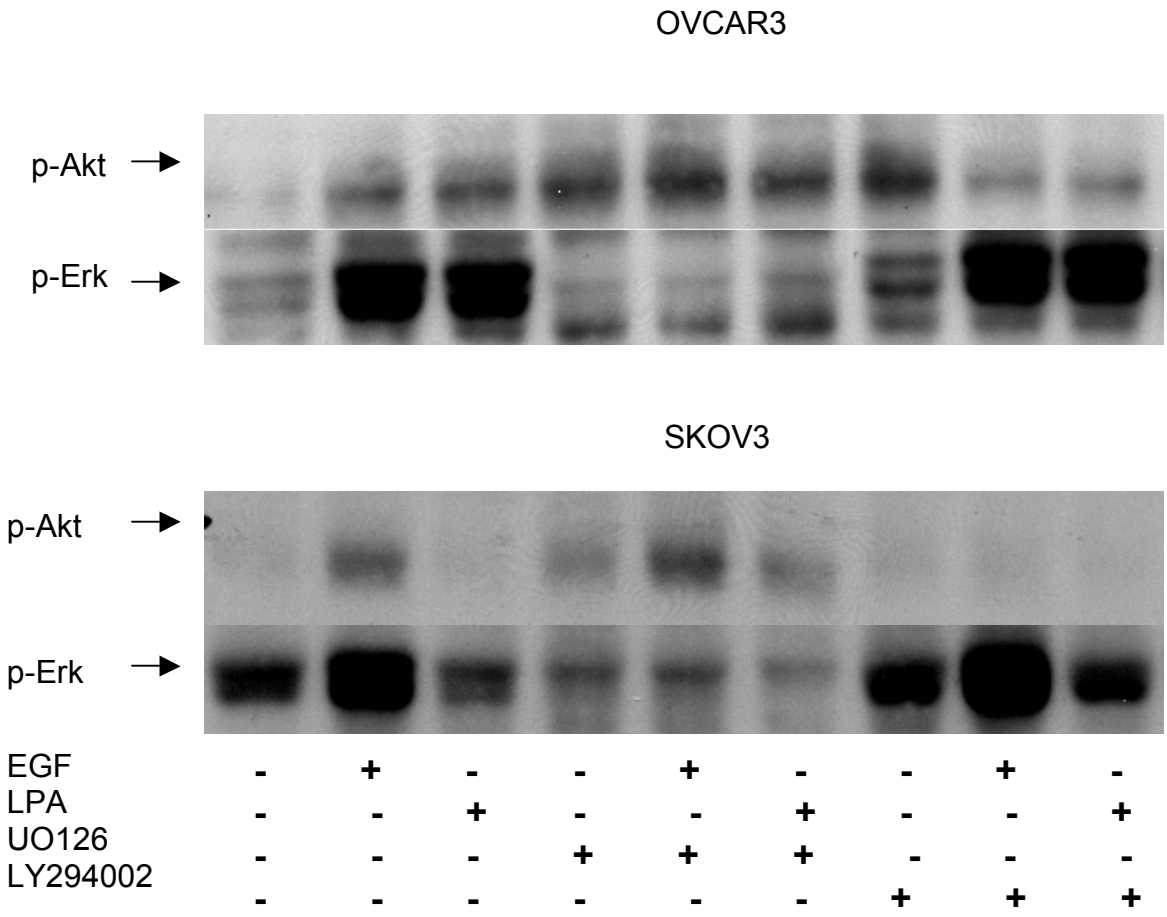


Figure 2-9. Effects of UO126 and LY294002 on intracellular signaling. OVCAR3 and SKOV3 cells were serum-starved for 12 hours. Cells were then pretreated with 10μM UO126 or 25 μM LY294002 for 5 minutes prior to stimulation with 10nM EGF or 10μM 18:1 LPA for 30 minutes. Whole cell extracts (100μg protein) were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were immunoblotted for phospho-Erk1/2 and phospho-Akt.

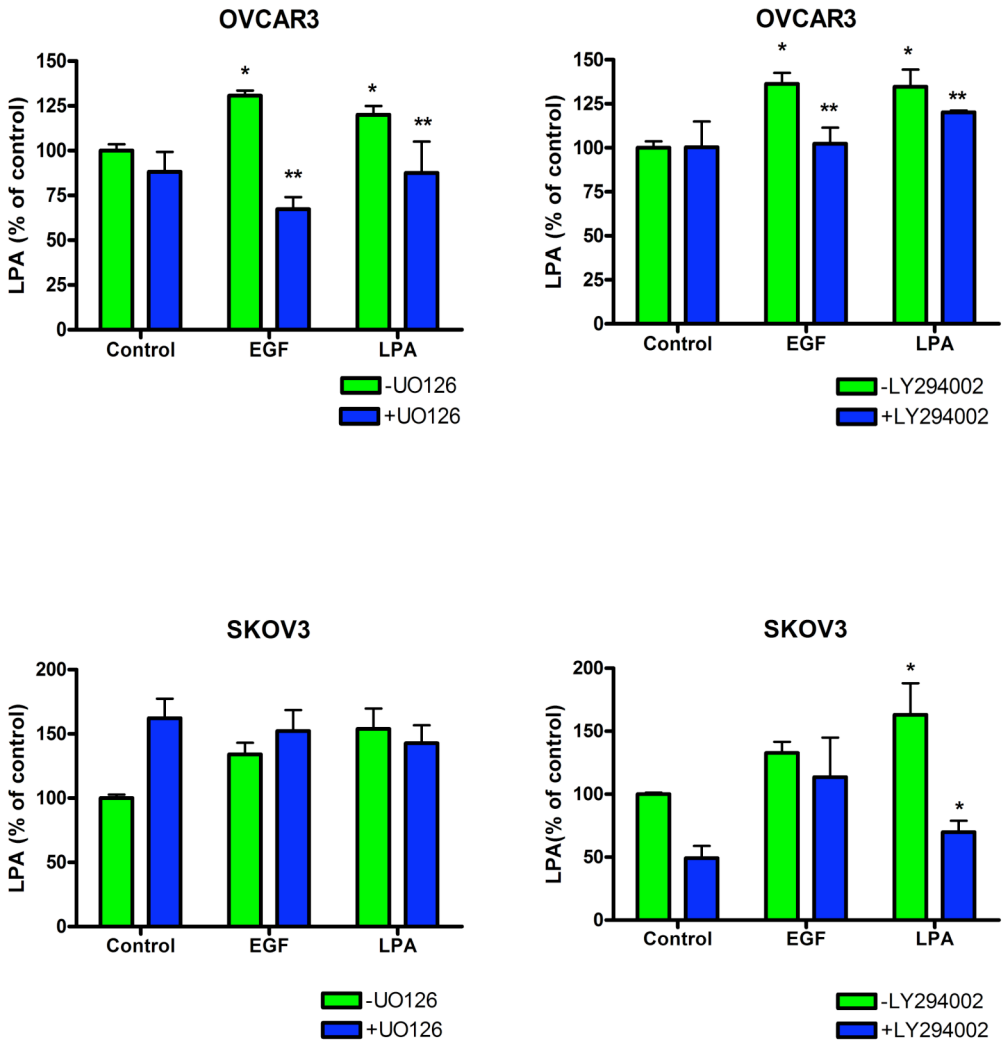


Figure 2-10. Effects of UO126 and LY294002 on LPA production. OVCAR3 and SKOV3 cells were metabolically labeled with [^{3}H]-palmitic acid for 12 hours and then pretreated with 10μM UO126 or 25 μM LY294002 for 5 minutes prior to stimulation with 10nM EGF or 10μM 18:1 LPA for 30 minutes. Medium was harvested and the lipids separated and quantified. The values expressed are normalized to the untreated controls and represent mean ± SEM of values from triplicate dishes of cells. (* represents treatment vs. control, ** represents treatment+inhibitor vs. treatment-inhibitor, $p<0.05$).

produced in SKOV3 cells, indicating a role for PI3K/Akt in LPA production as well. Together these data suggest that intracellular signaling molecules can play a role in agonist-induced LPA production, but do not provide a complete picture of the pathways involved due to the inconsistencies between cell lines.

2.5 DISCUSSION

Stimulatory effects of EGF and LPA on carcinoma cell growth, proliferation, migration and metastasis have been documented in numerous cancer cell types. LPA-induced transactivation of growth factor receptors, including EGFR (25, 90) and PDGFR (111, 134), has also been shown in various examples. LPA has been shown to stimulate EGFR transactivation through several mechanisms, including activation of MMPs and subsequent cleavage of pro-EGF forms (75), and via activation of intracellular kinases, such as c-Src (4). We show here that LPA can transactivate the EGFR in two ovarian cancer cell lines, OVCAR3 and SKOV3 (Figure 2-1).

This is the first study to show that EGF activates LPA production in ovarian cancer cells. This response can be blocked by an EGFR inhibitor (Figure 2-3A). Inhibition of the EGFR causes a decrease in both EGF- and LPA-stimulated LPA production. These data suggest that EGF alone can stimulate LPA production, and that EGFR transactivation plays a role in LPA-induced LPA production. LPA production by ovarian cancer cells has been shown to be stimulated by agonists such as phorbol 12-myristate 13-acetate (PMA) (114), and

also by agonists that activate the P2Y4 purinergic receptor (80). However, this is the first study to examine the role of EGF in LPA-induced LPA production, suggesting the role for crosstalk between the two receptors.

PTX has been shown to decrease numerous responses to LPA. LPA-induced Ca^{2+} mobilization (29), Ras Erk activation (73) , p38 MAPK activation (151), and nucleotide exchange (108) can be inhibited by PTX in various cell types. PTX blocked both EGF-and LPA-induced LPA production in OVCAR3 cells, indicating again that both receptors are necessary for LPA production in these cells. PTX acted similarly in SKOV3 cells, where EGF- and LPA-induced LPA production in SKOV3 cells were significantly inhibited by PTX. Ki16425, an LPAR antagonist, also decreased both EGF and LPA-induced LPA production in SKOV3 cells, further indicating bidirectional cross-talk between the receptors, and perhaps indicating that LPA-induced EGFR transactivation occurs via $G_{\alpha i}$ signaling. However, due to unexpected effects of the LPAR inhibitor Ki16425 in OVCAR3 cells, we were unable to assess the effects of LPAR inhibition on LPA production in these cells. In addition, since Ki16425 is a relatively new pharmacologic agent its specificity has not been completely established. Perhaps as knowledge of the LPA field advances, receptor antagonists be further refined, so that the role of LPAR transactivation can be further addressed.

Intracellular signaling cascades facilitate mitogenic responses elicited by both EGF and LPA. Two major phosphorylation cascades initiated by EGF and LPA are the Erk and Akt pathways. Both signaling pathways have been shown to increase proliferation of ovarian cancer cells (64, 96). When phosphorylation

of Erk was inhibited with UO126, LPA production was decreased in OVCAR3 cells (Figure 2-8). These data could suggest that EGF and LPA activate phospholipases downstream of Erks, such as cPLA2, that participate in LPA production. LY294002 decreased basal levels LPA production in SKOV3 cells, and inhibited EGF-and LPA-mediated LPA production in OVCAR3 cells, also implicating PI3K/Akt in agonist-induced LPA production. However, since consistent results were not observed between the two cell lines, this line of study was not further pursued.

We show for the first time that EGF and LPA both stimulate LPA production in two ovarian cancer cell lines. Inhibition of EGFR blunts the effect of both EGF and LPA on these responses. These data suggest that LPA-induced transactivation of the EGFR is necessary for LPA-induced LPA production. Given these findings, we suggest that the EGFR and LPARs signal bi-directionally, and that each types of receptor is necessary for the function of the other in agonist-induced LPA production. LPA-induced transactivation of the EGFR, and EGF-induced LPA production, signify cross-talk between the receptors.

CHAPTER III

ROLE OF AUTOTAXIN IN AGONIST-INDUCED LPA PRODUCTION

3.1 SUMMARY

Autotaxin (ATX) was first described as a motogen isolated from A-2058 human melanoma cells. ATX, initially identified as a phosphodiesterase, has been shown to be an extracellular form of lyso-PLD. ATX converts lysophosphatidycholine (LPC) to LPA extracellularly. ATX activity has proven difficult to detect at endogenous levels, with most assays using exogenous substrate or recombinant ATX. We show that ATX activity is present in culture medium from ovarian cancer cell lines. EGF can increase ATX activity in OVCAR3 cell medium, but only after 1 hour of treatment of with EGF. SKOV3 cells exhibited minimal basal levels of ATX activity; no stimulation of activity was seen even after 24 hours of EGF treatment. We show that ATX is distinct from a membrane form of lyso-PLD expressed in ovarian cancer cells. Small interfering RNA (siRRNA) for ATX was used to test the role of ATX in basal and agonist-induced LPA production in OVCAR3 cells. Stimulation of LPA production by either EGF or LPA was not affected following knockdown of ATX by siRNA. These results indicate that ATX does not play a major role in agonist-induced LPA production.

3.2 INTRODUCTION

Autotaxin (ATX), a motility factor and exophosphodiesterase (124), was first described to be released from A-2058 melanoma cells (124, 125). ATX was subsequently shown to have lyso-phospholipase D (lyso-PLD) activity (131). Extracellular ATX activity has been linked to increased motility (50), increased LPA production (38), and increased cellular proliferation (131). ATX is produced as a membrane-tethered exoenzyme. Upon cleavage from the membrane, ATX converts LPC to LPA.

ATX has been shown to participate in LPA production in numerous cell types including adipocytes (38), glioblastoma cells (67), and melanoma cells (9). The generation of LPA extracellularly allows for LPA to bind to its GPCR rapidly. Little is known about how much ATX contributes to the overall amount of LPA produced by tumor cells.

Studies demonstrating ATX lyso-PLD activity, typically use exogenous LPC added to the culture medium as a substrate (67). Over-expression of ATX in cells (65), or use of recombinant ATX (131) is often needed to demonstrate activity. There have been very few studies in which natural cellular conditions have been utilized.

It is not clear whether ATX activity can be stimulated or inhibited, either physiologically or pharmacologically. There are no known agonists or inhibitors of ATX or other lyso-PLDs. Therefore, we utilized siRNA for ATX to assess the influence of ATX activity on agonist-induced LPA production in OVCAR3 cells.

ATX siRNA was also used to determine if ATX accounts for membrane-associated lyso-PLD activity.

3.3 MATERIALS AND METHODS

Materials: Choline oxidase, 4-aminoantipyrene, $CaCl_2$, Tris-HCl, and horseradish peroxidase were from Sigma (St. Louis, MO). 18:1 LPA was from Avanti Polar Lipids (Alabaster, AL). ATX siRNA was custom designed and provided by Ambion (Austin, TX). BODIPY-LPC was from Molecular Probes (catalog # 3771) (Eugene, OR). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA).

Cell Culture: SKOV3 and OVCAR3 cells obtained from American Type Culture Collection (Manassas, VA) were maintained in RMPI 1640 medium (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), and McCoy's 5A medium supplemented with 10% fetal bovine serum. All cells were grown at 37°C in 5% CO_2/ 95% air on standard tissue culture plastic; cells were serum-starved by incubation in serum-free medium for 12-24 hours prior to experiments.

ATX activity assay: Cells grown in 6-well tissue culture plates were serum-starved for 12 hours prior to experimentation. The conditioned medium was retained on the cells during stimulation with agonists. Following incubation with agonists, the medium was harvested and subjected to centrifugation at 10,000xg

for 10 minutes to remove cellular debris. Cell culture medium (19µl) was then incubated with 1µM 18:1 LPC at 37ºC for 60 minutes. The color cocktail (180µl), containing 50mM Tris-HCl (pH. 8), 5mM $CaCl_2$, 0.3mM 4-aminoantipyrene, 5.3 units/ml horseradish peroxidase, and 2units/ml choline oxidase, was added to the sample and incubated at 37ºC for 20 minutes. Results were analyzed by spectroscopy at 550nM absorbance.

Incubation with siRNA for ATX: OVCAR3 cells were grown in 6-well plates to approximately 40% confluency. Cells were then incubated with siRNA for ATX according to the manufacturer's instructions. Briefly, cells were incubated in medium containing 10% FBS, along with 33pM ATX siRNA (final concentration) and Lipofectamine 2000 transfection reagent. Cells were incubated for 24-48 hours and then serum-starved for use in experiments.

In vitro Membrane Lyso-PLD Activity Assay*:* This assay is a modification of the membrane PLD assay described previously (140). Briefly, cells were incubated in serum free media for 12-24 hours and harvested. Cells were lysed by sonication. Membranes were separated using centrifugation at 100,000xg. Membranes (5µg protein) were incubated with BODIPY-lysophosphatidylcholine (B-LPC), in the absence of butanol, for 60 minutes at 30°. The samples were spotted on plastic-backed silica gel TLC plates and developed using methanol/chloroform/water/acetic acid (45:45:10:2 v/v). Products were visualized using UV light.

Isotopic Method for LPA analysis: LPA production in cells and medium was assessed as described previously (140). Briefly, cells were grown in 6-well plates and metabolically labeled with 5μCi/dish [^{3}H]-palmitic acid, in serum-free medium, for 12-24 hr prior to treatment. Cells were then incubated in Dulbecco's modified Eagle's medium supplemented with 10mM HEPES (pH 7.5) at 37°C for one hour. Agonists and/or antagonists were then added for varying periods of time. Following treatment, methanol/HCl and chloroform were used to extract lipids from cells and medium, as previously described (140). The lipids were dried under nitrogen and resuspended in chloroform/methanol. Oleoyl (18:1)-LPA was added to each sample as a standard. Thin-layer chromatography was used to separate the lipids. [^{3}H]-Labeled lipids were visualized using autoradiography after spraying the plates with Enhance (PerkinElmer Life Sciences. Inc., Boston, MA). The LPA band, as well as the other lipids in the lane, were separately scraped from the TLC plate and quantified using liquid scintillation spectrometry.

Statistical Analysis: All experiments were repeated; representative results are shown. For LPA production assays, the values shown are the mean ± SEM of values from triplicate dishes of cells. Statistical analyses were performed by one-way ANOVA using the InStat program.

3.4 RESULTS

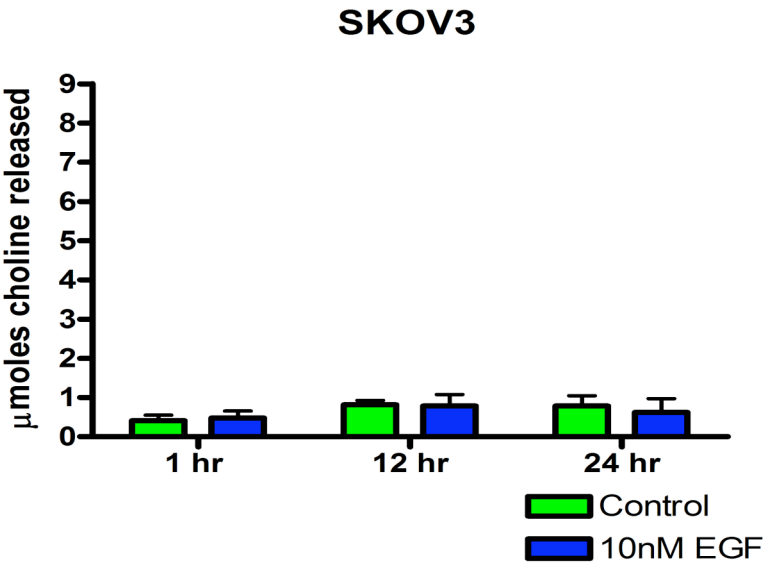
Autotaxin activity in ovarian cancer cells

An enzyme-based colorimetric assay (131) was adapted for use with cultured cells. Serum-starved cells were stimulated with 10nM EGF for 1, 12 or 24 hours; controls were also harvested for each of these time points. The medium was then tested for ATX activity. Control OVCAR3 cells displayed no dectable ATX activity at the 1 hour control time point (12hrs + 1 hr experiment) (Figure 3-1A), although activity was dectected after 48hrs (data not shown). The 12 and 24 hour time points did not show significant differences in the amount of ATX activity observed. EGF-treated OVCAR3 cells showed a significant increase in ATX activity after 1 hour incubation (again 12hrs +1hr). SKOV3 cells had very little ATX activity in any of the experiments shown (Figure 3-1B), although low activity was detected in the medium conditioned for 48 hours (data not shown).

Autotaxin and membrane lyso-PLD activity

Various forms of lyso-PLD have been described (141). Our laboratory has characterized a lyso-PLD activity present in mammalian membranes (Xie et. al., in preparation). This activity was detected using a fluorescent substrate.

A



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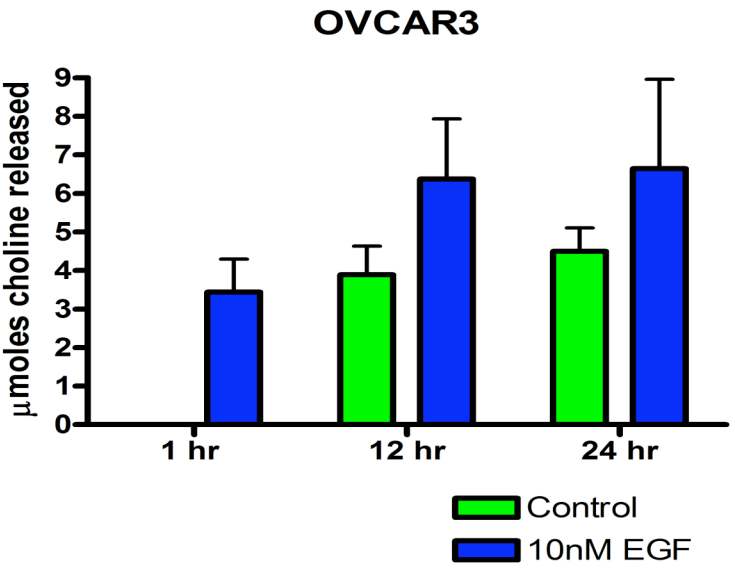

Figure 3-1. Autotaxin activity from ovarian cancer cells. OVCAR3 (A) and SKOV3 (B) cells were serum-starved for 12 hours. Cells were then retained in conditioned medium and incubated with or without 10nM EGF or 10µM 18:1 LPA for the indicated times. The data are normalized to the untreated controls and represent triplicate mean ± SEM of values from dishes of cells.

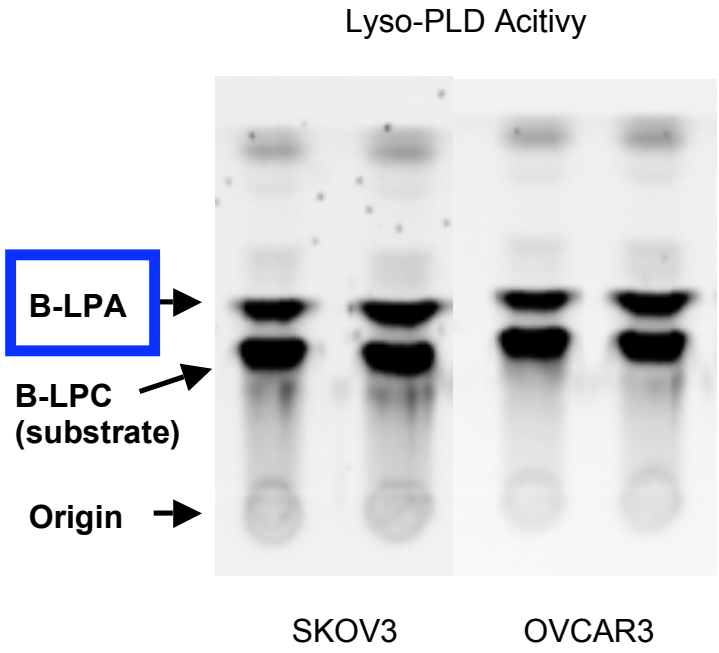

FIGURE 3-2. Basal membrane lyso-PLD activity in ovarian cancer cells. OVCAR3 and SKOV3 cells were harvested and membranes separated using sonication and centrifugation. Membrane samples (5µg protein) were incubated with BODIPY-LPC at 30°C for 60 minutes. Products were separated by TLC and visualized with fluorescence imaging. The B-LPA band is representative of lyso-PLD activity.

We used siRNA for ATX to down-regulate ATX activity in OVCAR3 cells. SKOV3 cells were not used due to their low ATX activity levels. OVCAR3 and SKOV3 cells both possess membrane lyso-PLD activity as detected by flourescent assay (Figure 3-3). OVCAR3 cells were incubated with 33pM siRNA for ATX, or with reagent alone, for 24-48 hours. Knockdown of ATX mRNA was validated by RT-PCR (Figure 3-3). Cells were then serum-starved for 12 hours and harvested. Membrane samples were prepared as described in Methods. BODIPY-LPC was incubated with membrane samples at 30°C for 60 minutes. Samples were separated by TLC and visualized with fluorescent imaging. Membranes from control, mock transfected, and ATX siRNA cells all generated LPA, indicative of membrane lyso-PLD activity (Figure 3-4). Membrane lyso-PLD activity remained even following knockdown of ATX mRNA, suggesting that membrane lyso-PLD activity is distinct from ATX. This finding is consistent with results obtained from the enzymatic characterization of membrane lyso-PLD (Xie et. al., in preparation).

Effects of siRNA for ATX on agonist-induced LPA production

To test the effect of ATX siRNA on LPA production, OVCAR3 cells were transfected with siRNA for ATX or with reagent alone. LPA production was assessed by the radiolabeling method described in Chapter II. Cells were treated with 10nM EGF or 10μM 18:1 LPA for 30 minutes and the medium was harvested.

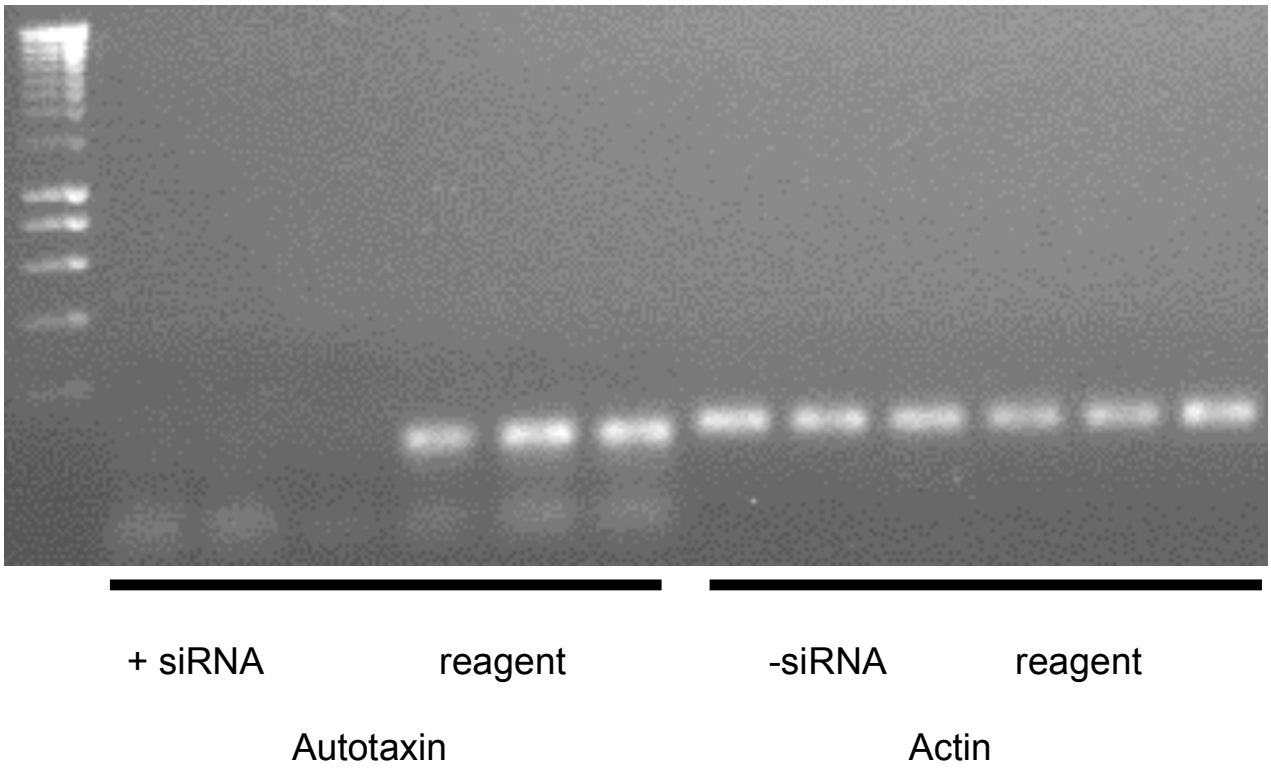

Figure 3-3. Effects of ATX siRNA on ATX mRNA levels in OVCAR3 cells. OVCAR-3 cells were incubated with Lipofectamine 2000 and 33pM siRNA for autotaxin, or with LF2000 alone, for 72 hours. Autotaxin and α-actin mRNAs were amplified by RT-PCR. Products were separated on an agarose gel containing ethidium bromide and visualized using UV light.

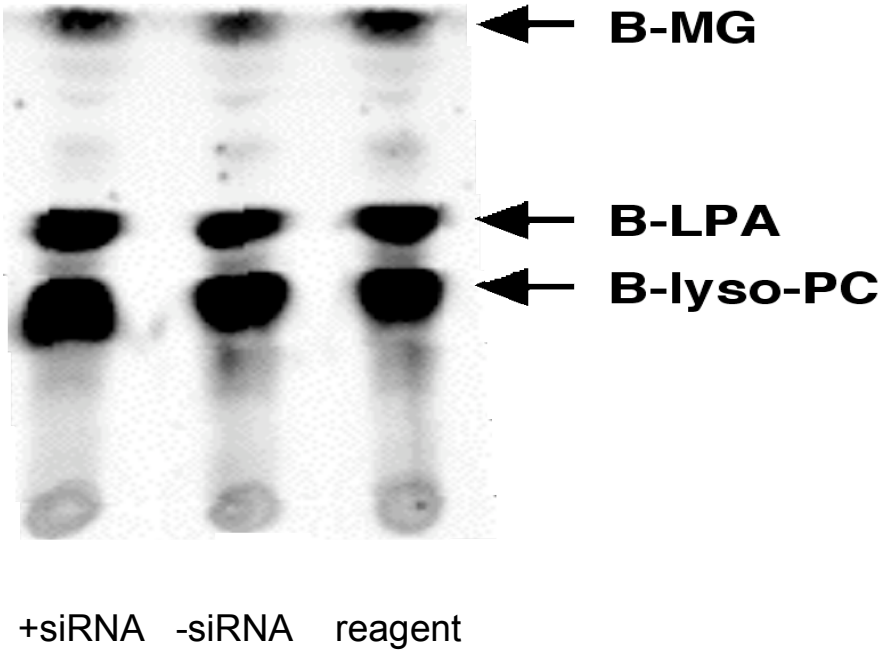

Figure 3-4. Effects of ATX siRNA on membrane lyso-PLD activity in OVCAR3 cells. OVCAR3 cells were incubated with LF 2000 and siRNA for ATX, without reagents, or with LF2000 alone for 72 hours. Membrane samples (5µg protein) were incubated with BODIPY-LPC for 60 minutes at 30ºC. Products were separated by TLC and visualized using fluorescence imaging.

Lipids were harvested and quantified. The siRNA for ATX did not significantly affect either EGF-or LPA-induced LPA production (Figure 3-5). The partial decrease in EGF-stimulated LPA production shown in Figure 3-5 was not significant, and was not consistent between experiments. These results suggest that ATX is not the primary enzyme responsible for agonist-induced LPA production under the experimental conditions examined.

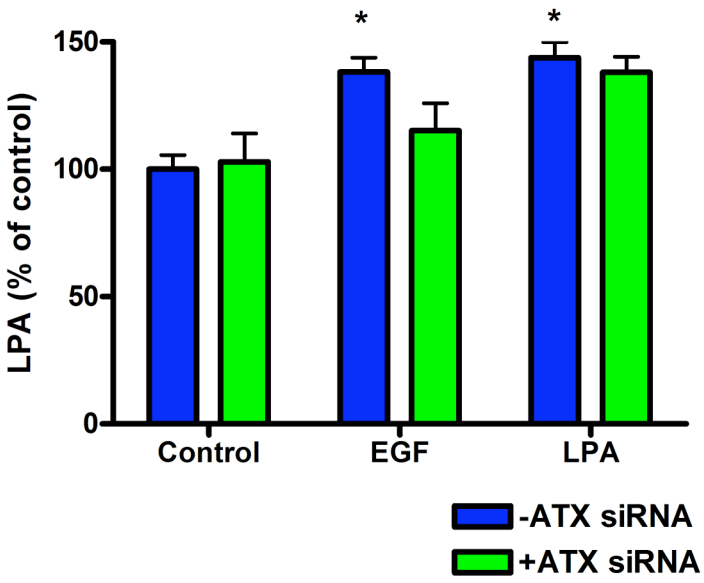

Figure 3-5. Effects of siRNA for ATX on LPA production from OVCAR3 cells. OVCAR3 cells were incubated with LF2000 and siRNA for ATX or LF2000 alone, for 72 hours. Cells were metabolically labeled using [^{3}H]-palmitic acid in serum-free medium. After washing, cells were incubated with or without 10nM EGF or 10μM 18:1 LPA for 30 minutes. Medium was harvested and the lipids separated and quantified. The values expressed are normalized to the untreated controls and represent mean ± SEM of values from triplicate dishes of cells.

3.5 DISCUSSION

LPA appears to accumulate in the extracellular space of carcinoma cells, such as in the ascites fluid surrounding ovarian tumors (137). How LPA accumulates in the extracellular space is still unknown. LPA may be produced within the membrane and exported out of the cell, or be produced extracellularly. ATX, an extracellular enzyme, can produce LPA outside cells. It has therefore been hypothesized that ATX is responsible for LPA accumulation extracellularly. Here we have shown that ATX activity is present in both OVCAR3 and SKOV3 cells (Figure 3-1). However, ATX activity is low under the conditions used. This is consistent with the fact that the cells were washed prior to the assay; this step would be expected to remove accumulated extracellular ATX. These conditions were used to match those of the LPA production assay used in our lab. An increase in extracellular ATX activity occurs only after 1 hour incubation with 10nM EGF in OVCAR3 cells. No increase could be detected in SKOV3 cells. These results suggest that ATX activity is not responsible for the increases in LPA production that are observed within 30 minutes after agonist addition. ATX may contribute to basal levels of LPA production, but does not appear to be involved in agonist-induced LPA production in ovarian cancer cells.

To further test whether EGF-or LPA-induced LPA production was mediated by ATX, we used siRNA to decrease ATX expression levels. We found that membrane lyso-PLD activity is not affected by ATX siRNA. Membrane lyso-PLD, like ATX, has not been shown to be activated by agonists; this topic will be

discussed further in Chapter IV. Using siRNA to down-regulate ATX had no apparent effect on agonist-induced LPA production. These data support the conclusion that ATX is not involved in agonist-induced LPA production by these two cell lines.

While ATX does not seem to be responsible for agonist-induced LPA production, there could still be other roles for ATX in ovarian cancer cells that we did not examine. ATX was first discovered as a motogen (70), and may effect motility and/or the metastatic potential of ovarian cancer cells. In addition, since the relative contributions of basal and agonist-stimulated LPA production to overall LPA levels are unknown, ATX or other lyso-PLDs may play a major role in total LPA production. Having excluded a role for ATX in agonist-induced LPA production, we explored the roles of other enzymes (Chapter IV).

CHAPTER IV

ROLE OF PHOSPHOLIPASE D2 IN AGONIST-INDUCED LPA PRODUCTION

4.1 SUMMARY

Phospholipases are critical regulators of cellular processes. Lyso-phospholipase D (lyso-PLD), and phospholipase D (PLD) are enzymes that can be involved in LPA production. PLD and lyso-PLD cleave choline from cellular phospholipids and lysophospholipids, respectively. PLD exists in two isoforms, PLD1 and PLD2. Both PLD isoforms have been shown to be agonist regulated. A role for secreted lyso-PLD (autotaxin) in agonist-induced LPA production was excluded in the previous chapter. In this study we investigated which pathways are most important for LPA production by ovarian cancer cells. Both OVCAR3 and SKOV3 cells have basal PLD and lyso-PLD activity, and PLD2 activity is stimulated by both EGF and LPA in OVCAR3 cells. Neither agonist stimulates membrane lyso-PLD activity. PLD2 overexpression increases basal PLD activity and LPA production. Small interfering RNAs (siRNAs) were used to lower levels of PLD2 in both OVCAR3 and SKOV3 cells. Agonist-induced LPA production in OVCAR3 cells is inhibited by siRNA for PLD2. These data implicate PLD2 in agonist-induced LPA production in OVCAR3 cells.

4.2 INTRODUCTION

Phospholipase-mediated lipid metabolism influences cellular processes, and changes cellular dynamics. Phospholipases are responsible for production of LPA. Agonists can regulate some of these enzymes, but how this regulation affects agonist-induced LPA production is still unknown. Two phospholipases potentially involved in LPA production are PLD and lyso-PLD.

PLD and lyso-PLD are localized to the membrane that can potentially participate in LPA. Lyso-PLDs cleave choline from lysophosphatidylcholine (LPC) to form LPA. One membrane form of this enzyme has been characterized in our lab, and has no known agonist. PLD, which cleaves choline from phosphatidylcholine (PC), exists in two isoforms, PLD1 and PLD2. Both isoforms of PLD hydrolyze PC to form PA, but are regulated differently. PLD1 (46) can be activated by small GTPases, such as Rho and ARF, as well as by PKC and PIP2 (51, 117). PLD2 has been shown to interact with the EGFR (118), and can be activated by PIP2 (103). PLD2 co-localizes with the EGFR when over-expressed in HEK293 cells (62, 118), but it is not known if this is occurs under physiological conditions.

PLA_2 cleaves side chains from the *sn2* position of phospholipids, resulting in formation of lysophospholipids, such as lysophosphatidylcholine (LPC). PLA_2 exists in numerous isoforms. Calcium-independent ($iPLA_2$) and secretory PLA_2 ($sPLA_2$) enzymes can both cleave fatty acid chains at the *sn2* position of the glycerol backbone, but are not known to be agonist regulated. Cytosolic PLA_2

(c PLA_2) is activated upon phosphorylation by ERKs. Both $cPLA_2$ and $iPLA_2$ have been implicated in PMA-stimulated LPA production by ovarian cancer cells (114). In this cited study, alcohol was used to divert the PLD reaction to produce phosphatidylalcohol, rather than PA. This resulted in decreased LPA production. This approach is problematic in that primary alcohols have other cellular effects. PLA_1 and PLA_2 isoforms are involved in forming lysophospholipids. However, it is unknown which of these enzymes are involved in EGF-or LPA-stimulated LPA production.

The role of PLD and lyso-PLD in basal and agonist-induced LPA production in ovarian carcinoma cells was the focus of this study. We examined basal levels of both PLD and lyso-PLD activities. We used membrane-based assays to determine if EGF or LPA activates either enzyme. We utilized over-expression siRNA, and pharmacologic inhibitors to determine the role of PLD2 in LPA production. The results implicate PLD2 in agonist-induced LPA generation.

4.3 MATERIALS AND METHODS

Materials: BODIPY-PC and -LPC were from Molecular Probes (Eugene, OR), siRNAs for PLD2 were from Ambion (Austin, TX). Phosphatidylethanol and phosphatidic acid were supplied by Avanti Polar Lipids (Alabaster, AL). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA).

Cell Culture: SKOV3 and OVCAR3 cells. obtained from American Type Culture Collection (Manassas, VA), were maintained in RMPI 1640 medium (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), and McCoy's 5A medium supplemented with 10% fetal bovine serum. All cells were grown at 37°C in 5% CO_2/ 95% air on standard tissue culture plastic and serum-starved by incubation in serum-free medium for 12-24 hours.

In vitro Membrane PLD Activity Assay: Serum-starved cells were incubated with the desired agonist, and then harvested at 4ºC. Cells were lysed by sonication; membranes were collected by centrifugation at 100,000xg. Cellular membranes were incubated with 9% butanol and BODIPY-phosphatidylcholine (B-PC) for 60 minutes at 30° as previously described (140). The samples were spotted on plastic-backed silica gel TLC plates and developed with methanol/chloroform/ water/acetic acid (45:45:10:2 v/v). Products were visualized using UV light.

Intact Membrane PLD Activity Assay: Cells were metabolically labeled with [^{3}H]-palmitic acid (5µCi/dish) for 12-24 hr in serum-free medium prior to treatment. Prior to treatment, cells were washed twice with Dulbecco's modified Eagle's medium supplemented with 10mM HEPES (pH 7.5) and incubated at 37°C in this medium for one hour. Cells were harvested following treatment with or without agonists in the presence of 0.5% ethanol, lipids were extracted using methanol/HCl and chloroform, as previously described (140). The lipids were

dried under nitrogen. Phosphatidic acid (PA) and phosphatidylethanol (PEt) were added to each sample as standards. The samples were loaded on a TLC plate, which was developed using ethyl acetate/acetic acid/water (90:20:100 v/v). The plate was exposed to x-ray film. Bands were scraped, and PLD activity quanitfied following liquid scintillation spectrometry of PA, PEt, and the remainder of the lipid. Data are expressed as a percent of total lipid recovered, for normalization.

In vitro Membrane Lyso-PLD Activity Assay: Cells were incubated in serum-free medium for 12-24 hours, then incubated with the desired agonist and harvested. Cells were lysed by sonication; membranes were separated using centrifugation at 100,000xg at 4ºC. Cellular membranes were incubated with BODIPY-lysophosphatidylcholine (B-LPC) for 60 minutes at 30°. The samples were spotted on plastic-backed silica gel TLC plates and developed using methanol/chloroform/water/acetic acid (45:45:10:2 v/v). Products were visualized using UV light in a fluorescence imaging system (Molecular Dynamics).

Transfection with PLD2 Expression Vector: Cells were seeded and grown to approximately 40% confluence. Cells were then transfected with an HA-tagged PLD2 over-expression vector (Knoepp et. al., in preparation) using Lipofectamine 2000 LF2000 according to the manufacturer's instructions. Briefly, cells were transfected for 48 hours with 1µg of DNA using LF2000 and serum-starved for 12 hours prior to experimentation.

Incubation with siRNA for PLD2: Cells were seeded and grown to approximately 40% confluence. Cells were then incubated with pre-designed siRNAs for PLD2 from Ambion, according to the manufacturer's directions. Briefly, cells were incubated with 20nM siRNA for PLD2, using LF2000, for 48 hours. Cells were then serum-starved prior to experimentation.

Isotopic Method for LPA analysis: LPA production in cells and medium was assessed as described previously (140). Briefly, cells were grown in 6-well plates and then metabolically labeled with 5μCi/dish [^{3}H]-palmitic acid, in serum-free medium, for 12-24 hr prior to treatment. The cells were washed and incubated in Dulbecco's modified Eagle's medium supplemented with 10mM HEPES (pH 7.5) at 37°C for one hour. Agonists and/or antagonists were then added for varying periods of time. Following treatment, methanol/HCl and chloroform were used to extract lipids from cells and medium, as previously described (140). The lipids were dried under nitrogen and resuspended in chloroform/methanol. Oleoyl (18:1)-LPA was added to each tube as a standard. Thin-layer chromatography was used to separate the lipids. [^{3}H]-labeled lipids were visualized using autoradiography after spraying the plates with Enhance (PerkinElmer Life Sciences. Inc., Boston, MA). The LPA band, as well as the other lipids in the lane, were separately scraped from the TLC plate and quantified using liquid scintillation spectrometry.

4.4 RESULTS

Basal and stimulated levels of PLD and lyso-PLD

We first examined whether lyso-PLD or PLD2 were activated by agonists in ovarian cancer cells. OVCAR3 and SKOV3 cells were incubated for 15 minutes with or without agonist (10nM EGF or 10μM 18:1 LPA), and then harvested. Membrane samples were incubated with either 0.9% butanol and either BODIPY-PC, or BODIPY-LPC, for 60 minutes at 30°C. Products were then separated by TLC and visualized by phospho-imaging. Both OVCAR3 and SKOV3 cells displayed basal levels of PLD and lyso-PLD activity (Figure 4-1). The PLD assay has been shown to be specific for PLD2 activity under the conditions used (140). Both EGF and LPA stimulated PLD2 activity in OVCAR3 cells. In SKOV3 cells no visible stimulation was seen with either EGF or LPA; however, this lack of effect could potentially be due to lack of sensitivity of the assay. Lyso-PLD activity was not consistently stimulated by EGF or LPA in either cell line.

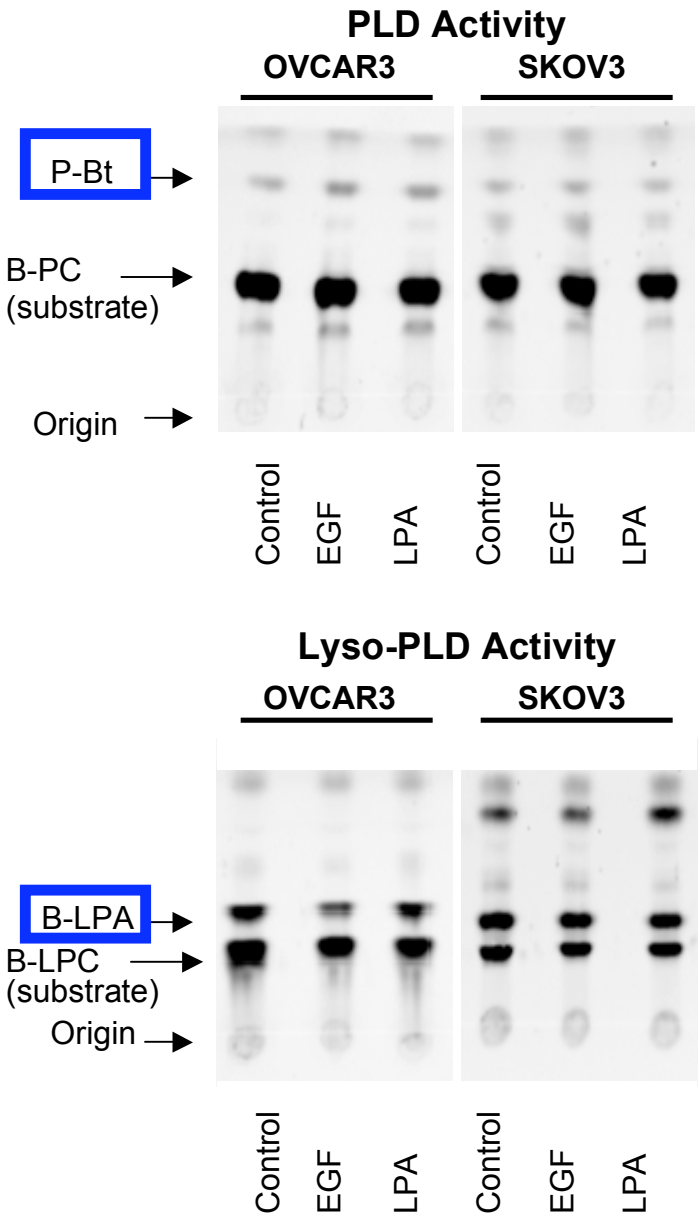

Figure 4-1. Basal and stimulated PLD and lyso-PLD activities in ovarian cancer cells. OVCAR3 and SKOV3 cells were serum starved for 12 hours and then treated with 10nM EGF or 10μM 18:1 LPA for 15 minutes. Cells were harvested and membranes separated by sonication and centrifugation. A. Membrane samples were incubated with 9% butanol and BODIPY-PC at 30°C for 60 minutes. B. For both panels, membrane samples were incubated with BODIPY-LPC at 30°C for 60 minutes. Products were separated by TLC and visualized using fluorescence imaging.

Effect of overexpression of PLD2 on PLD activity and LPA production

PLD2 was over-expressed in OVCAR3 cells in order to assess the role of PLD2. An expression vector encoding HA-tagged PLD2 was transfected into OVCAR3 cells using LF2000 for 48 hours. Cells were then serum starved; PLD activity was assessed using both an intact cell PLD activity assay and an *in vitro* PLD activity assay.

PLD2 over-expression in OVCAR3 cells increased PLD activity measured in the membrane PLD assay (Figure 4-2A.) PLD2 activity was stimulated in OVCAR3 cells treated with 10nM EGF for 15 minutes, and was also increased in cells over-expressing PLD2. However, EGF did not further increase membrane PLD2 activity in cells over-expressing PLD2.

In an intact cell assay (Figure 4-2B), EGF stimulated total PLD activity. Basal activity was increased in OVCAR3 cells over-expresing PLD2. EGF increased PLD activity in cells over-expressing PLD2, but the total level of activity was not higher than seen in untransfected cells. Thus, both the membrane and intact cell assays suggest that there is a maximal level of PLD2 activity that can be achieved.

We next examined the effects of PLD2 over-expression on LPA production. PLD2 was over-expressed in OVCAR3 cells. Cell labeled with [^{3}H]-palmitic acid were then incubated with 10nM EGF or 10μM 18:1 LPA for 15 minutes. Medium was harvested; LPA production was assessed as described in

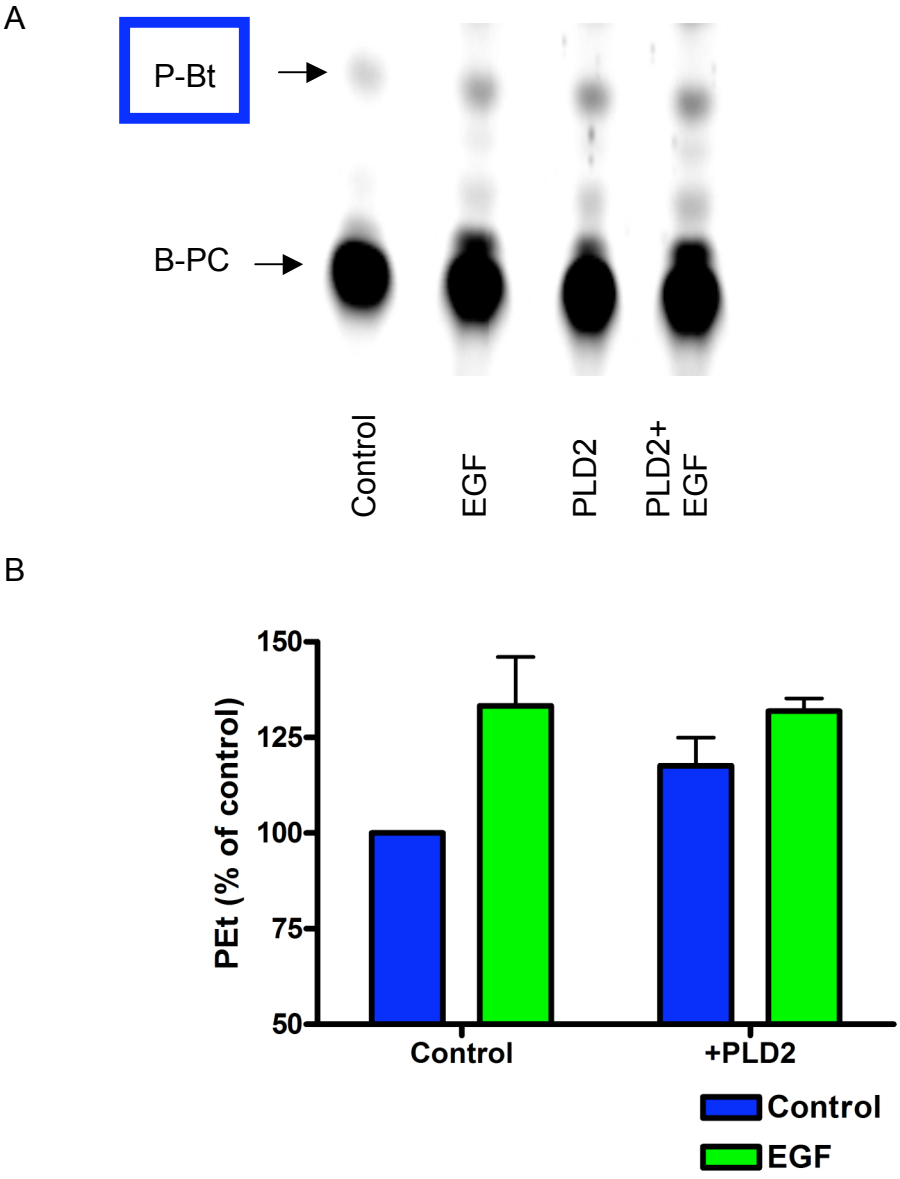

Figure 4-2. Effects of PLD2 over-expression on PLD activity. OVCAR3 cells were transfected with PLD2 overexpression vector for 48 hours. A. Cells were serum-starved for 12 hours. Following stimulation, with or without 10nM EGF, cells were harvested. Membranes were separated and incubated with BODIPY PC and 9% butanol. PLD products were separated by TLC and visualized by phospho-imaging. B. Cells were serum-starved and labeled with [^{3}H]-palmitic acid. After washing cells were incubated with 0.5% ethanol and with or without 10nM EGF for 15 minutes. Cells were harvested and lipids were separated using TLC. PEt was quantified using liquid scintillation spectrometry as a measure of PLD activity. The values expressed are normalized to the untreated controls and represent mean ± SEM from triplicate dishes of cells.

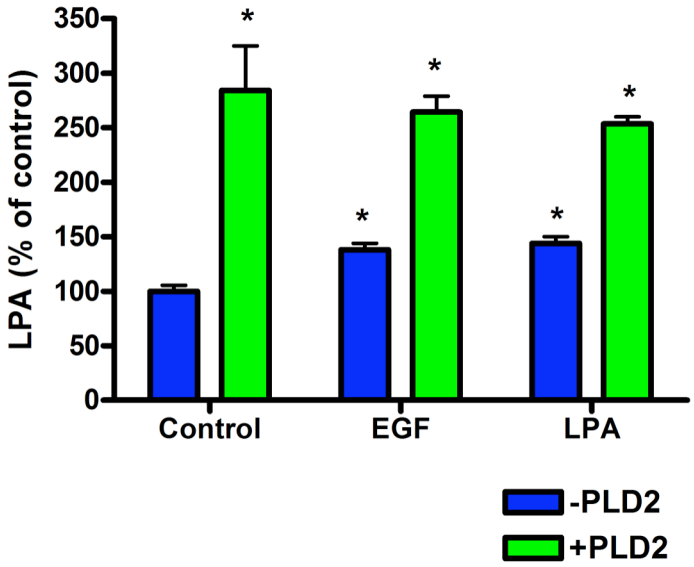

Figure 4-3. Effect of PLD2 over-expression on LPA production in OVCAR3 cells. OVCAR3 cells were transfected with a PLD overexpression vector for 48 houres. Cells were serum-starved and metabolically labeled with [^{3}H]-palmitic acid for 12 hours. Cells were then treated with 10nM EGF or 10μM 18:1 LPA for 30 minutes. Medium was harvested, lipids were separated using TLC and quantified using liquid scintillation counting. The values expressed are normalized to the untreated controls and represent triplicate dishes of cells. (*represents PLD2 over-expressing vs. control, $p<0.01$)

Chapter II. Over-expression of PLD2 increased basal LPA production in OVCAR3 cells (Figure 4-3). LPA and EGF increased LPA production in control cells, as shown in Chapter II. However, agonists did not cause a further increase in LPA production in cells over-expressing PLD2. Thus, there appears to be a limit to the amount of LPA that can be produced by cells.

Effect of siRNA for PLD2 on LPA production in ovarian cancer cells

To further assess the role of PLD2 in agonist-induced LPA production, we used a knockdown approach. OVCAR3 and SKOV3 cells were incubated with 20nM siRNA for PLD2 plus LF2000, with 20nM non-silencing siRNA plus LF2000, or with LF2000 alone for 48 hours prior to serum-starvation. Serum-starved cells were then harvested and membranes prepared. In cells transfected with PLD2 siRNA, membrane PLD activity was markedly decreased (Figure 4-4); transfection with reagent alone or with non-silencing siRNA had no effect. These results confirm that transfection with PLD2 siRNA reduces membrane PLD2 activity. Attempts to further confirm knockdown of PLD2 mRNA were unsuccessful due to poor results with PCR primers.

We next assessed the effects of PLD2 siRNA on LPA production by OVCAR3 and SKOV3 cells. Both cell lines were incubated with siRNAs for PLD2 plus LF2000, or with LF2000 alone, for 48 hours. Cells were metabolically labeled with [^{3}H]-palmitic acid for 12 hours in serum-free medium. After washing, cells were incubated with or without 10nM EGF or 10μM 18:1 LPA for 30 minutes. In SKOV3 cells, there was a trend toward decreases in agonist-induced

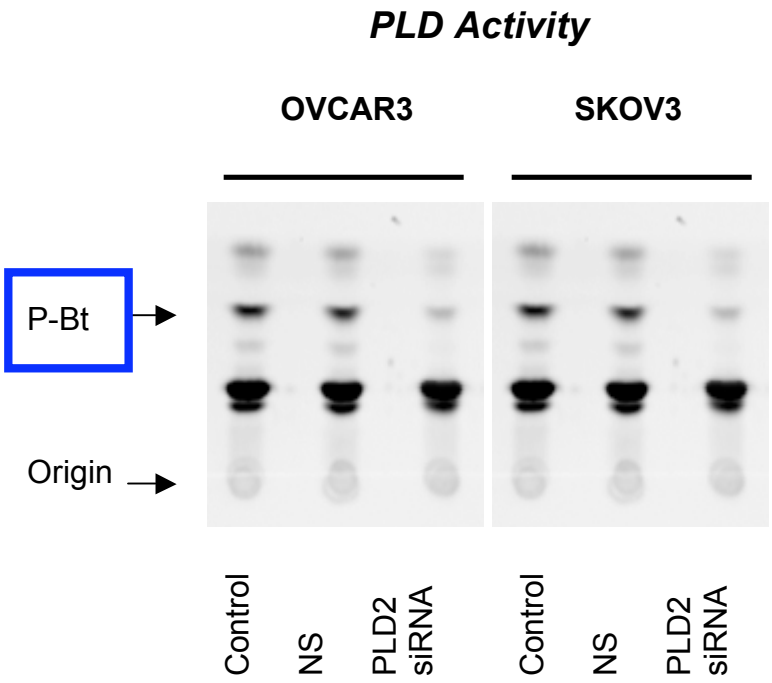

Figure 4-4. Effects of siRNA for PLD2 on membrane PLD activity in ovarian cancer cells. OVCAR3 and SKOV3 cells were transfected with 20nM siRNA for PLD2, non-silencing siRNA, or reagent alone for 48 hours. Cells were serum-starved and harvested after 12 hours. Membranes were separated with sonication and centrifugation. Membrane samples were incubated with 9% butanol and BODIPY PC for 60 minutes at 30°C. PLD products were separated by TLC and visualized by phospho-imaging.

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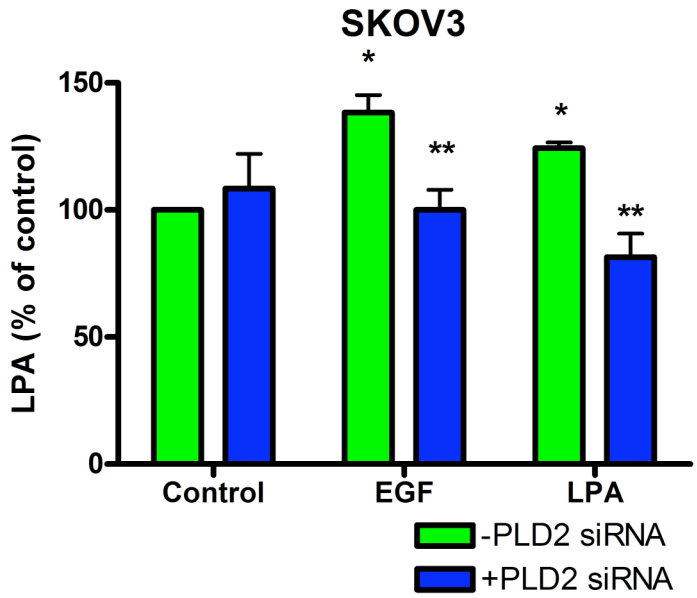

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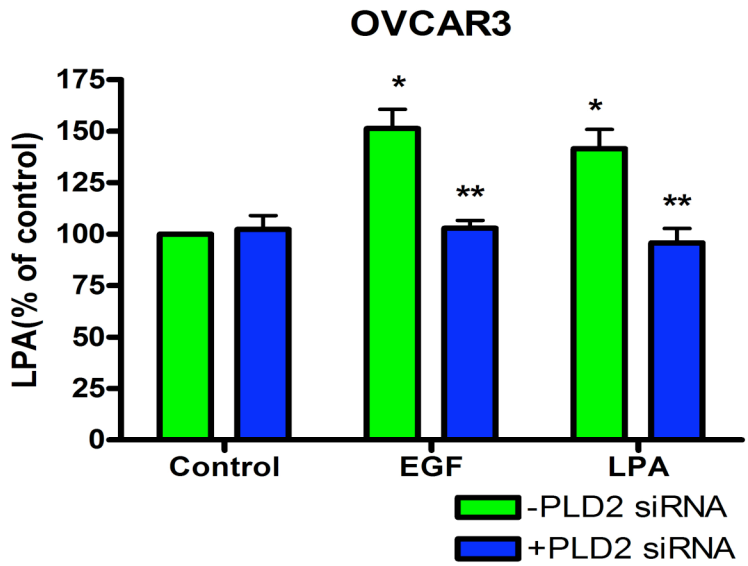

Figure 4-5. Effects of siRNA for PLD2 on LPA production by ovarian cancer cells. OVCAR3 and SKOV3 cells were transfected using Lipofectamine 2000 with siRNA for PLD2, or LF2000 alone, for 48 hours. Cells were metabolically labeled using [^{3}H]-palmitic acid in serum-free medium. After washing, cells were incubated with or without 10nM EGF or 10μM 18:1 LPA for 30 minutes. Medium was harvested and the lipids separated and quantified. The values expressed are normalized to the untreated controls and represent mean ± SEM for six experiments. One asterisk represents $p < 0.01$ for agonist vs. control value; two asterisks represent $p<0.01$ for PLD2 siRNA vs vehicle (t-test).

LPA with PLD2 siRNA, but statistical significance was not achieved due to variability in control values.

In OVCAR3 cells, PLD2 siRNA decreased both EGF-and LPA-induced LPA production, albeit with a significant decrease only for EGF-induced LPA production (Figure 4-5). These data implicate PLD2 in agonist-induced LPA production in OVCAR3 cells.

4.5 DISCUSSION

PLD and lyso-PLD activities exist in the membrane of cells. PLD activity has been characterized in numerous mammalian cell lines(46). PLD has been shown to be activated by small GTPases, receptor tyrosine kinases, and phosphoinositides (36, 102, 103), while membrane lyso-PLD has no known regulators. We show here that EGF and LPA simulate PLD2 activity, but not membrane lyso-PLD activity.

In this study we show that PLD2 activity can be stimulated by both EGF and LPA in OVCAR3 ovarian cancer cells. Over-expression of PLD2 leads to maximal activity of PLD in OVCAR3 cells (Figure 4-2), accompanied by a maximal increase in LPA production (Figure 4-3). These findings suggest a role for PLD2 in LPA production.

PLD was implicated in LPA production in SKOV3 cells, by another group of investigators, during the course of this dissertation project (80). However, this published study did not link EGF or LPA to PLD-mediated LPA production. In

Figure 4-5 we show that knockdown of PLD2 activity blunts agonist-induced LPA production in OVCAR3 cells. These data suggest that EGF and LPA can stimulate LPA production through PLD2 activation.

EGF has been shown to complex with PLD2, as well as to phosphorylate PLD2 (118). Dissociation of Munc-18-1, an inhibitory protein, from PLD2 in response to EGF facilitates PLD2 activation (77). Protein kinase C (PKC) has also been shown to mediate EGF stimulation of PLD (148). Numerous signaling molecules including phospholipase C (PLC) (59) and PKC (23, 148) have been shown to regulate EGF-mediated PLD2 activation, both positively and negatively. Future studies could be directed at determining the pathway by which EGF activates PLD2 in OVCAR3 cells to mediate LPA production.

CHAPTER V

GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 GENERAL DISCUSSION

EGFRs and LPARs induce a multitude of intracellular signaling cascades. These signaling cascades lead to growth, proliferation and migration of cancer cells (56, 151). Separately, agonists for these receptors can promote tumor growth. However cross-talk between receptors is likely to contribute to mitogenic effects. Specifically, LPA can transactivate the EGFR, enlisting additional signaling pathways to amplify mitogenic responses. Over-expression of EGFR is common in cancers, including 35-70% of ovarian cancers (41, 109). While the density of LPARs may not be as high as that of EGFRs, when LPA binds to its receptors it can potentially induce the shedding of numerous pro-EGF ligands. Acting in concert, LPA and EGF can potentially activate a plethora of intracellular signaling pathways both singularly and together.

In this dissertation we show that EGF can induce LPA production, further adding to the cross-talk between receptors. This cross-talk results in a positive-feedback loop between the EGFR and LPARs. EGF can induce LPA production, leading to LPAR activation and potentially EGFR transactivation. These findings could lead to a therapeutic target to prevent the cross-talk. In Chapter II, we show that by blocking EGFR signaling we can block both EGF- and LPA-induced LPA production. These results strengthen the idea of receptor cross-talk, as opposed to transactivation. We also show that both Erk and Akt are potentially involved in agonist-induced LPA production. It has previously been reported that Akt and Erk are needed for LPA-induced proliferation (151), but this is the first

time that the signaling molecules have been implicated in LPA production. These data suggest a role for the EGFR and LPARs, as well as intracellular signaling to play a role in agonist-induced LPA production in ovarian cancer cells.

Breast cancer therapeutics have already been targeting the EGFR (150). The data from Chapter II suggest that EGFR could be a target in ovarian cancer therapy as well. Over-expression of EGFR in ovarian cancer has already been documented (41, 109). In accordance with the data from this chapter, these therapies could potentially block EGF-mediated signaling, as well as LPA induced signaling, in ovarian cancer.

Another potential target for ovarian cancer therapy could be the ligand for the EGFR instead of the EGFR itself. As with the naturally occurring insulin-like growth factor binding proteins (IGF-1 BPs), the therapeutic could be directed toward sequestering the EGFR ligands. Admittedly this would not likely be successful for all EGFR ligands; however, currently HB-EGF is being considered as an important target for ovarian cancer therapy (85, 145). HB-EGF has been shown to be released from numerous types of tumors (39, 104, 145), and is thought to be the primary EGFR ligand released upon LPA activation of MMPs (85, 104). Inhibition of HB-EGF would decrease some effects induced by both LPA and EGF. However, since EGFRs will bind other ligands, this approach might not block all EGF-mediated responses.

Autotaxin (ATX), an extracellular enzyme, has been shown to produce LPA (131). As we show in Chapter III, ATX does not account for the EGF-induced

LPA production in either OVCAR3 or SKOV3 cells. We used siRNA for ATX to confirm that EGF-mediated LPA production is not generated by ATX. When over-expressed, or in the presence of excess substrate, ATX can produce enough LPA to initate cellular responses. Nonetheless, as we have shown, there is not enough endogenous ATX activity to account for agonist-induced LPA production. Due to differences in assay conditions between laboratories, it is not yet clear what portion of the total bioactive LPA is produced by agonist-induced LPA production.

PLD2, which hydrolyzes phosphatidylcholine (PC) to phosphatidic acid (PA), has been shown to be involved in LPA production in ovarian cancer cells (80). Nucleotide agonists acting through a P2Y4 purinergic receptor have been shown to stimulate PLD2 activity, and to increase LPA production in SKOV3 cells (80). EGF has also been shown to bind to and phosphorylate PLD2 (59, 118). Complexing between phospholipase C (PLC) and PLD2 has been shown to be important for downstream signaling of EGFR (59). PLD2 has been shown to be activated by growth factors (134), and to mediate LPA production in SKOV3 cells (80). We show for the first time that EGF-mediated LPA production in OVCAR3 cells involves PLD2.

PLD2 is involved in numerous cellular processes and can mediate cellular adhesion, as well as cellular signaling (2, 102). We demonstrate in this dissertation that PLD2 can be activated by EGF and LPA in ovarian cancer cells. This activation then leads to increased LPA production, lending PLD2 to potentially become a cancer therapeutic target.

Therapeutic inhibition directed at the synthesis of lipid mediators, and at their receptors, has already proven to be effective at treating disease states such as asthma. Leukotrienes (LTs), products of arachidonic acid metabolism, facilitate symptoms of asthma, such as bronchoconstriction, bronchial smooth muscle hyperresponsiveness, and inflammatory cell recruitment (27). Arachidonic acid is converted to 5-HPETE by 5-lipoxygenase (5-LO) and further by leukotriene synthases to various forms of leukotrienes. Current 5-LO inhibitors, such as zileutin, block leukotriene formation, resulting in less inflammation in asthma (27). Montelukast and zafirlukast, inhibit leukotriene receptors (LTRs), and block LT-mediated inflammatory responses. These same concepts could be applied to therapeutic antagonists for LPARs, as well as inhibitors of the enzymes involved in LPA production, such as PLD2.

Currently, there are no pharmacologic inhibitors of PLD1 or PLD2. However, there are pharmacologic inhibitors for proteins involved in activation of PLD. EGFR inhibition has been extensively studied. Inhibition of the tyrosine kinase activity of EGFR can be achieved with either Iressa or Tarceva(53, 58). These two molecules could inhibit EGFR-induced PLD activation. LPA-induced activation of PLD could be inhibited using an LPAR antagonist. In combination, and EGFR inhibitor and a LPAR antagonist could block both EGF- and LPA-induced PLD activation of PLD.

Based on our findings that EGFRs and LPARs act in concert, and that both activate the PI3K/Akt pathway, another potential therapeutic approach would be to combine EGFR and/or LPAR inhibitors with and Akt inhibitor. The

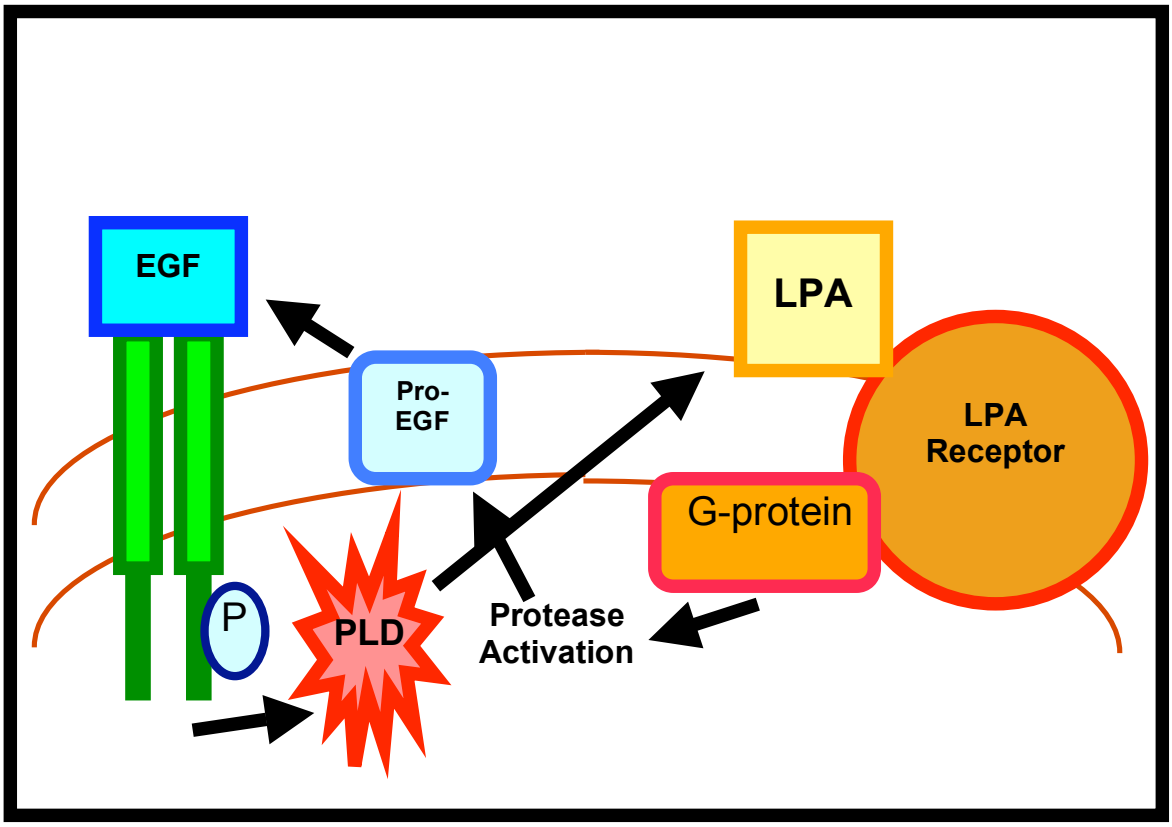

FIGURE 5-1. Receptor cross-talk and a role for PLD2 in agonist-mediated LPA production. LPA can bind to its receptor and induce transactivation of EGFR, as well as inducing its own production. Here we have shown that EGF binding to its receptor can activate LPA production. This model suggests that PLD2 is activated by EGF to increase LPA production in OVCAR3 cells

therapeutic use of combinations of signal transduction inhibitors is still in its infancy, but could result in a synergistic blockade of cancer cell growth and survival.

In this dissertation we have shown that EGFRs and LPARs are involved in receptor cross-talk, and that this cross-talk is necessary for agonist-induced LPA production. We have shown that agonist-induced LPA production is facilitated by PLD2 in OVCAR3 cells. Future studies should examine the roles of PLD2 in other EGF- and LPA-mediated responses.

5.2 FUTURE DIRECTIONS

In this dissertation we explored cross-talk between EGFRs and LPARs. However, we did not explore which LPAR is most influential in this process. LPARs can be promiscuous in binding to G-proteins, therefore inducing various signaling pathways. It has been shown that LPA1 couples to $G_{12/13}$, while LPA2 couples to $G_{\alpha i}$, and LPA3 couples to $G_{\alpha q}$. Coupling of LPARs to mutilple G-proteins, have been documented. LPA_1 has been shown to play a regulatory role in cell growth (133). LPA_2 has been shown to be associated with transformation of normal cells to cancerous phenotypes (68, 116). LPA_2 can also mediate mitogenic signaling in colon cancer cells (149). The role of LPA_3 has not been fully elucidated. Future studies could utilize siRNA to down-regulate specific

LPARs, singly and in combination, to determine the role of each receptor in agonist-induced responses in ovarian cancer cells.

Two enzymes that were not explored in this dissertation, but potentially could be involved in LPA production by ovarian cancer cells, are acylglycerol kinase (AGK) and PLD1. AGK, a mitochondrial enzyme, has been shown to phosphorylate monoacylglycerol to generate LPA in prostate cancer cells (13). Suppression of AGK with siRNA has been shown to block EGF-mediated signaling in PC-3 cells (122). We were able to demonstrate that both cell lines express AGK mRNA (data not shown). We attempted to down-regulate AGK in OVCAR3 and SKOV3 cells, using several siRNAs, but were unsuccessful. The role of AGK in agonist-induced LPA production could be important. Our study focused solely on PLD2, an enzyme that we have shown to be activated by EGF and LPA. However, PLD1 may likewise be involved in LPA production. PLD1 could also be knocked down using siRNA to determine its role in agonist-induced responses.

The mechanism of dual cross-talk between EGFRs and LPARs is worthy of further examination. EGFRs have been shown to form a complex with the angiotensin receptor (AT_1) (95). Such an interaction could potentially occur between the EGFR and LPARs. Immunoprecipitation experiments could be conducted to determine if these receptors form a complex, and begin to examine whether such an interaction is necessary for activity of one or both receptors.

In addition to receptor complexes, the role of PLD2 in such complexes could be explored. PLD has been shown to associate with the EGFR (118), but the role of this complex is still not fully understood. PLD siRNAs could be used to determine the potential role of PLD in receptor complexes between EGFRs and LPARs, if such complexes are demonstrated. The role of PLD2 catalytic activity in LPA production should be addressed. Is PLD2 needed to form PA, leading to LPA, or is PLD2 critical for some other aspect of EGF/LPA signaling? This is an important issue that remains to be addressed.

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Oral Presentations:

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2. **Jones A.C.,** and Meier K.E., Lysophosphatidic Acid (LPA) Production by Ovarian Carcinoma Cells is Stimulated by Epidermal Growth Factor (EGF) **,** 7th Annual P/T Presentation Day, Washington State University, 2004.

3. **Jones A.C.,** Zhang Z., and Meier K.E., Signal Transduction, Lipids and Reproductive Cancers, NWRSS, Portland, OR, April 2006.

Poster Presentations:

1. **Jones A.C.,** and Meier K.E., Epidermal Growth Factor (EGF) Stimulates Lysophosphatidic Acid (LPA) Production in Prostate and Ovarian Cancer Cells, 17:4, FASEB, EB 2003.

2. **Jones A.C.,** and Meier K.E., Lysophosphatidic Acid (LPA) Production is Stimulated by Epidermal Growth Factor (EGF) in Prostate and Ovarian Cancer Cells, FASEB Summer Conference on Phospholipids, 2003.

3. **Jones A.C.,** and Meier K.E., Epidermal Growth Factor (EGF) Promotes Lysophosphatidic Acid (LPA) Production in Ovarian Carcinoma Cells, FASEB, EB, 2004.

4. **Jones A.C.,** and Meier K.E., Lysophosphatidic Acid (LPA) Production by Ovarian Carcinoma Cells is Stimulated by Epidermal Growth Factor (EGF), Southeastern Regional Lipid Conference, 2004.

5. **Jones A.C.,** Xie Y., Meier K.E., Pathways Involved in EGF-Stimulated Lysophosphatidic Acid (LPA) Production in Ovarian Carcinoma Cells, 8th Annual P/T Presentation Day, Washington State University, 2005.

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8. Brauner D.J., Zhang Z., **Jones A.C**., Rubio M.V., and Meier K.E.: Lysophosphatidic Acid (LPA) Protects Against Calpain-Mediated Degradation of Focal Adhesion Kinase (FAK) in Human Prostate and Ovarian Cancer Cells, FASEB, EB 2006.

9. **Jones A.C.,** and Meier K.E., Involvement of PLD2 in Lysophosphatidic Acid Production by Ovarian Carcinoma Cells, Southeastern Regional Lipid Conference, 2006.

Articles:

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2. Park J.H., Um T., Xie Y., Knoepp S.M., Brauner D., **Jones A.C**., Rubio M.V., Gibbs T.C., Meier K.E., Lysophosphatidic Acid Inhibits Calpain-mediated Proteolysis of Focal Adhesion Kinase in Prostate Cancer Cells, in preparation.

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