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MOLECULAR MECHANISMS FOR REGULATION OF GENE EXPRESSION BY LYSOPHOSPHATIDIC ACID IN OVARIAN CARCINOMA CELLS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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List of Abbreviations

AA	Arachidonic acid
Ab	Antibody
AP-1	Activator Protein-1
ATF	Activating transcription factor
ATP	Adenosine triphosphate
ATX	Autotaxin
Bcl10	B-cell CLL lymphoma 10
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
C-terminus	Carboxyl terminus
C/EBP	CCAAT enhancer binding protein
CamK	Camodulin kinase
cAMP	Cyclic adenosine monophosphate
CARMA3	CARD and MAGUK domain-containing protein 3
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
Cox	Cyclooxygenase
CRE	cAMP response element

DMEM	Dulbecco's modified eagle medium
DN	Dominant negative
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDG	Endothelial differentiation gene
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked immuno sorbent assay
EMSA	Electromobility shift assay
ERK	Extracellular signal-regulated kinsae
FBS	Fetal bovine serum
Fra	Fos-related antigen
G	Guanine nucleotide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GPCR	G protein coupled receptor
GSK-3	Glycogen synthase kinase-3
h	hour
HDAC	Histone deacetylase

HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
IκB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
IL	Interleukin
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kDa	Kilo Dalton
LAP	Liver activating protein
LIP	Liver inhibiting protein
LPA	Lysophosphatidic acid
MALT-1	Mucosa associated lymphoid tissue lymphoma translocation gene 1
MAPK	Mitogen-activated protein kinase
min	minute
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor-kappa light chain enhancer of B cells
N-terminus	Amino- terminus
PAF	Platelet-activating factor
PAGE	Polyacrylamide gel electrophoresis
PAP	Potato alkaline phosphatase
PCR	Polymerase chain reaction

PGE_2	Prostaglandin E2
PI3K	Phosphoinositol 3-kinase
PKC	Protein kinase C
PKD	Protein kinase D
PPAR	Peroxisome proliferator-activated receptor
PTX	Pertussis toxin
RHD	Rel homology domain
RLU	Relative luciferase unit
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSK	p90 ribosomal S6 kinase
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
S1P	Sphingosine-1-phosphate
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
siRNA	Small interfering ribo nucleic acid
TBK1	TANK-binding kinase 1
TK	Thymidine kinase
TNF-α	Tumor necrosis factor alpha
TRE	12-*O*-tetradecanoylphorbol-13-acetate (TPA) responsive element

uPA	Urokinase plasminogen activator
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WT	wildtype
Y	Tyrosine

Abstract

MOLECULAR MECHANISMS FOR REGULATION OF GENE EXPRESSION BY LYSOPHOSPHATIDIC ACID IN OVARIAN CARCINOMA CELLS

By Regina Adenike Oyesanya, PhD

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

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Lysophosphatidic acid (LPA) is a potent bioactive phospholipid mediator that functions through multiple G protein couple receptors (GPCRs). LPA is elevated in ascites of ovarian cancer patients and is involved in growth, survival and metastasis of ovarian cancer cells. Gene promoter analyses revealed that some LPA-target genes share similar sets of binding sites for prominent transcription factors posing the possibility of a general mechanism for activation of their expression by LPA. Detailed investigation of the

mechanisms of regulation of cyclooxygenase 2 (Cox-2), a paradigm of LPA-regulated genes, showed that LPA robustly upregulated the expression of Cox-2 in ovarian cancer cells through multiple receptors. LPA induced rapid increase in Cox-2 mRNA and significantly enhanced the stability of Cox-2 transcript with the support of mRNA binding protein HuR. The effects of LPA on Cox-2 transcriptional activation include essential involvement of transcription factor, C/EBP-β. Further studies on mechanisms of activation of C/EBP-β demonstrated that LPA increased phosphorylation, binding and transcriptional activities of C/EBP-β. In addition, activation of C/EBP-β and LPA-target genes required contribution from EGFR. This novel crosstalk between LPA GPCRs and EGFR in mediating transcription factors activation was further explored by investigating the mechanisms of activation of AP-1 and NF-κB by LPA. Activation of AP-1 family of proteins by LPA relied heavily on basal inputs from EGFR as inhibition of EGFR kinase activity with AG1478 caused significant loss of LPA-induced AP-1 expression, binding and transcription activities. Although HGF and other agonists of RTK only weakly stimulate LPA-target genes and transcription factors in ovarian cancer cells, costimulation with HGF in the presence of AG1478 restored LPA signals to both C/EBP-β and AP-1. This suggests an obligatory role for a RTK in LPA-induced transcriptional activation, not necessarily inputs from EGFR. Interestingly, inhibition of EGFR with AG1478 did not interfere with LPA-induced NF-κB activation. Pharmacological inhibition and molecular targeting revealed that only a subset of G proteins participate in the crosstalk between LPA receptors and EGFR. Collectively, these results demonstrate the presence of at least two

signals downstream of LPA receptors: one dependent on basal RTK activity and another mediated directly by LPA GPCRs.

CHAPTER ONE

INTRODUCTION

1.0 OVERVIEW

Cancer is a disease of uncontrolled cell growth. Cancer cells often possess the ability to invade adjacent tissues through the release of substances or molecules that can degrade the tissue material. Some of these malignant cells may also spread to distant sites in the body via the blood or lymph, a process known as metastasis. All aspects of cancer development, survival and progression are strongly anchored on abnormal gene expression, a consequence of bypass of critical points of gene regulation. Many cancer types originate from cells that possess gene mutations, deletions or amplifications. As such, certain cellular functions including activation of transcription factors or posttranslational modifications of synthesized proteins become highly enhanced. Tumor-suppressing genes are inactivated while growth-promoting oncogenes are continually turned on, giving the cells new properties, particularly growth advantages. Major mechanisms responsible for the enhanced growth of malignant cells include reduce dependence on growth factor, insensitivity to growth inhibition, evasion from programmed cell death (also called apoptosis), and unlimited growth potentials. The adverse effects of cancer on patients are therefore mainly due to tumor burden from increased cell number

from the dysregulated cell growth. Most cancer therapies target the reduction of cell number and the prevention of further accumulation of tumor cells.

Ovarian cancer remains the leading cause of death from gynecological cancer and the fifth leading cause of death from cancer in women. According to the American Cancer Society, there are an estimated 21,650 new cases of ovarian cancer and 15,520 deaths due to this malignancy in the United States in 2008 [1]. Ovarian epithelial carcinoma is the most common type of ovarian cancer. This type includes endometrioid carcinoma, serous carcinoma, mucinous carcinomas, clear cell carcinoma and borderline tumor. Ovarian cancer is classified into four stages (Stages I-IV). Most tumor markers are insufficient to detect ovarian cancer at early Stages I and II because they either lack sensitivity and specificity for ovarian cancer or are not elevated until advanced stages of the disease. Hence, ovarian cancer has been termed a "silent killer" due to of the lack of symptoms or accurate tumor markers at the early stages leading to poor prognosis.

At the advanced Stages III and IV, ovarian cancer is often characterized by extensive intraperitoneal distribution of tumors and formation of large volumes of ascitic fluid. The ascitic fluid from ovarian cancer patients contains the ovarian tumor cells and a broad range of potent growth factors including lysophosphatidic acid (LPA) [2, 3]. The levels of LPA in the plasma of ovarian cancer patients, including those at early stages, are significantly higher than those in normal controls [2, 4]. Up to 80 μM LPA has been found in the ascitic fluid of ovarian cancer patients [4-6]. LPA is therefore considered to be biomarker for ovarian cancer. It is now known that LPA influences many processes of tumor cells including growth, survival migration and metastasis [7, 8]. LPA mediates these

processes at least partially through regulating the expression of diverse genes and metabolic pathways [9-12]. Understanding the detailed mechanisms by which LPA regulates gene expression may lead to identification of critical therapeutic targets for the treatment of ovarian cancer and perhaps, other cancer types.

1.1 Metabolism of LPA

LPA (1-acyl-*sn*-glycerol-3-phosphate) is a naturally-occurring phospholipid. It can be produced by different cell types including activated platelets [13, 14], endothelial cells [15], fibroblasts [16], adipocytes [17], prostate [18] and ovarian cancer cells [7]. Thus, it is present in body fluids including plasma (bound to albumin), saliva, hair follicles and malignant effusions [19, 20]. There are multiple pathways potentially responsible for the endogenous generation of LPA particularly the actions of certain enzymes on phospholipids of the cell membranes [13, 21]. A major part of LPA produced by activated platelets is synthesized by the sequential actions of phospholipase A_1 or A_2 ($PLA_{1/2}$) on serum or membrane phospholipids such as phosphatidylcholine (PC) followed by hydrolytic actions of a lysophospholipase D (lysoPLD) present in plasma (Fig. 1.1). Recently, autotaxin (ATX), an exo-phosphodiesterase, implicated in cell motility was found to be an important enzyme in the production of LPA and the predominant source of extracellular LPA [19, 22]. ATX is synthesized as a full-length, or pre-pro-enzyme, that is proteolytically cleaved in transit along the classical export pathway and secreted as a catalytically active glycoprotein [23]. With its intrinsic lysoPLD activity, ATX can

hydrolyze lysophosphatidylcholine (LPC), a major phospholipid secreted by hepatocytes and therefore abundant in blood and plasma [24-26], into LPA. The phosphorylation of monoacylglycerol by acylglycerol kinase (AGK) is another source of LPA [18]. When overexpressed, AGK in the mitochondria is able to mediate the production and secretion of LPA by phosphorylation of monoacylglycerol. The exact pathways for the generation of LPA in ascites, saliva, seminal and other body fluids are yet to be fully delineated.

Similarly, the mechanism for intracellular production of LPA is poorly understood. In ovarian and other cancer cells, LPA production can be stimulated by cell activation in response to phorbol esters [27], bombesin [27] and LPA itself [28, 29]. The activation of LPA production may involve multiple steps catalyzed by phospholipases, unlike the extracellular pathway where the precursors of LPA already preexist. In normal cells, the intracellular and extracellular LPA levels are tightly controlled by LPA synthesizing and metabolizing enzymes. Lipid phosphate phosphohydrolases (LPP) are a family of enzymes that catalyze the dephosphorylation of LPA [30-32]. These enzymes are membrane-associated with extracellularly-facing catalytic site for clearance of LPA on the cell membrane. There is evidence that expression of these enzymes reduce LPA levels and compromise LPA-induced cellular functions [30]. In addition to dephosphorylation, LPA can also be converted to phosphatidic acid (PA) by acylation through the action of LPA acyl transferases (LPAAT) [33, 34].

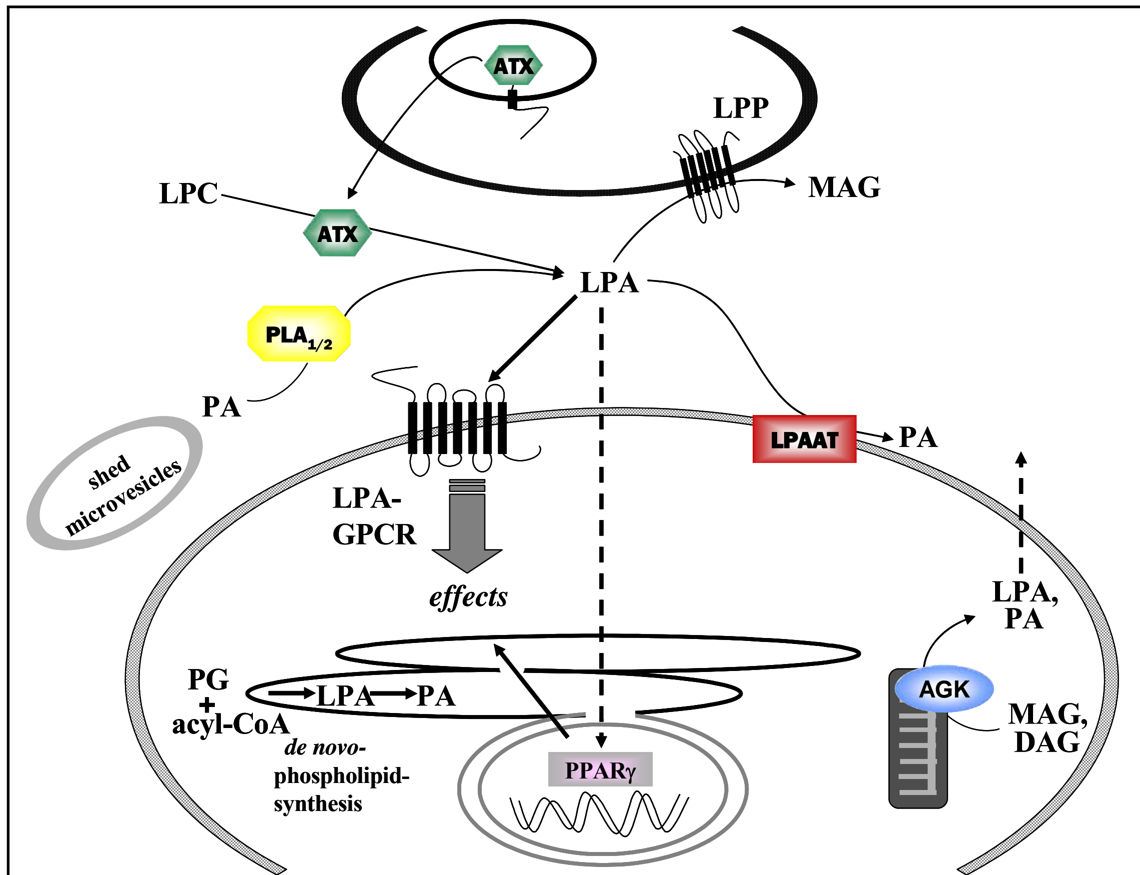


Fig. 1.1. Metabolism of bioactive LPA.
LPA is formed extracellularly by diverse pathways including the deacylation of phosphatidic acid (PA) by $PLA_{1/2}$ and the cleavage of lysophospholipids, predominantly LPC by autotoxin, which represent the major source of extracellular LPA. Overexpression of AGK in mitochondria was recently shown to promote the generation and release of LPA from monoacylglycerol (MAG) and diacylglycerol (DAG). [After **Biochim Biophys Acta. 2007: 1768(4):923-40**]

1.2 LPA Receptors and Signal Transduction

LPA is a bioactive phospholipid and a potent mediator of a broad range of cellular responses. It promotes cell proliferation and survival; enhances cell migration and invasion; and induces changes in actin cytoskeleton and focal contact organization [3, 7,

29]. These responses culminate from the activation of a diverse array of signaling pathways initiated when LPA binds its receptors on the plasma membrane. At least seven LPA receptors have been identified. Based on their primary structure, LPA receptors are classified into two groups: the endothelial differentiation gene (Edg) group and the purinergic receptor family (P_2Y) group. LPA_1/Edg-2, LPA_2/Edg-4 and LPA_3/Edg-7 belong to the Edg family and share about 50-57% homology in their amino acids [35-38]. LPA_4/$P2Y_9$/GPR23 and LPA_5/P_2Y_5 of the P_2Y family of receptors are two novel LPA receptors structurally distant from the LPA receptors of the Edg family, sharing only 20-24% homology with $LPA_{1\text{-}3}$ [39, 40]. LPA has also been identified as a ligand for two additional orphan receptors GPR87 and P_2Y_{10} of the P_2Y family [41, 42]. The identities of these receptors as bona fide LPA receptors are yet to be thoroughly studied.

LPA receptors are G protein coupled receptors (GPCRs). They elicit their activities by coupling to trimeric G proteins subunits, G_α and $G_{\beta\gamma}$ [35-40]. Aberrant regulation GPCRs have been linked to numerous diseases including cardiovascular defects, diabetes, allergies and certain forms of cancer [43-46]. More than 30% of the drugs in current use target the inhibition of GPCRs [47, 48]. LPA GPCRs couple to diverse G proteins including G_i, G_q and $G_{12/13}$ to initiate the activation of parallel yet interactive intracellular signaling cascades culminating in physiological responses. Activation of G_q mediates the activation of phospholipase C (PLC) with subsequent hydrolysis of phosphatidylinositol biphosphate (PIP2) to inositol trisphosphate (IP3), an activator of intracellular calcium release and diacylglycerol (DAG), that activates protein kinase C (PKC) [16, 49]. Gi mediates the inhibition of adenylate cyclase leading to downregulation of intracellular

cAMP. G_i or associated $G_{\beta/\gamma}$ subunit are also linked to activation of Ras and downstream mitogenic Ras/mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) [50, 51]. Activation of Ras-MAPK and PI3K are critical to LPA-induced cell-proliferation, migration and survival [50, 52]. The effects of LPA on stress fibre formation and the cell cytoskeleton occur through the activation of $G_{12/13}$/ RhoA [53].

Rapid internalization of receptor from the plasma membrane following ligand-induced activation is one way of quenching LPA signals [54]. The mechanism of the metabolic fate of the receptor after internalization is not yet known. In addition to GPCRs, LPA may also have some intracellular targets including proliferator-activated receptor γ (PPAR-γ) [55, 56]. PPAR-γ regulates the transcription of genes involved in glucose and fatty acid metabolism, adipocytes differentiation and inflammation process [57, 58]. LPA may be able to enter the cell in sufficient quantity and activate this intracellular receptor, suggesting its participation in intracellular signaling and cell functions. While overexpression studies have helped to understand the general functions of LPA receptors, the challenge remains as to the assignment of LPA receptor subtypes to specific signal transduction cascades and define their relative contribution to the multiple biological activities of LPA.

1.3 Role of LPA in Tumor Biology

Gene targeting and pharmacological inhibition of LPA receptor subtypes in mice and different cell types revealed diverse physiological and pathological roles for LPA

signaling. The Edg LPA receptors are differentially expressed in various tissues [59, 60]. LPA_1 is most widely expressed and present in both normal and malignant cells. In contrast, expression of LPA_2 is more restricted. LPA_3 is barely seen in normal tissues [60]. Recent studies showed that, in ovarian and thyroid cancers, malignant transformation is associated with increased expression of LPA_2 (and LPA_3 in ovarian cancer) [61, 62]. LPA receptors are also overexpressed in many other cancer types including endometrioid, colon, and colorectal cancer [63-65]. These observations suggest that changes in LPA receptor expression during malignant transformation are intimately involved in carcinogenesis. Furthermore, increased expression LPA receptors also correlates with important cancer progression processes such as migration and metastasis in many cancer types [63-65]. Recent studies have demonstrated that the presence of LPA in intraperitoneal effusions of ovarian cancer patients may contribute significantly to the progression and aggressive characteristics of the malignant cells [7, 62, 66]. In addition, various ovarian cancer cell lines respond to LPA stimulation with increase in migration and invasion. The influence of LPA on various cellular processes is supported by its ability to regulate the expression of diverse genes.

1.4 Regulation of Gene Expression

The most fundamental task of any organism is the control of the expression of the thousands of genes harbored by its genome. Genes are segments of DNA that carry information necessary for the development and proper functioning of all living organisms.

A normal cell possesses the capacity to process in parallel, the many regulatory inputs received from within and without, into enormous regulatory outputs that are tissue specific. When a cell grows, it divides by replicating its DNA into two daughter cells, each having the same genetic information as the parent cell. Gene expression, the representation of this inheritable information from the sequence of bases of the DNA to functional forms, is a complex and tightly regulated process. Diverse functions and features are acquired by selectively expressing or repressing segments of the DNA. Although basal expression of certain genes occurs in a resting cell, the active expression or repression of many genes are signaled for by complex sets of molecules and processes within or external to the cell. Abnormal gene expression by malignant cells is a result of the circumvention of these regulatory signals.

Microarray analysis of LPA-induced gene expression in an ovarian cancer cell line from our lab showed that LPA stimulated expression of many cancer-related genes (Table 1.1). LPA can therefore modulate cellular responses of malignant cells by inducing expression of these targets genes including cytokines, proteases, cell adhesion molecules, proangiogenic factors and anti-apoptotic genes. The array of LPA-target genes continue to expand ascribing new roles for LPA in more physiological and pathological contexts. However, the mechanistic details of how LPA regulates the expression of many genes remain elusive. As described in Chapter 2 of this dissertation, we have focused on LPA-induced expression of cyclooxygenase 2 (Cox-2) as a model to elucidate the mechanisms of gene regulation by LPA.

Table 1.1 Microarray analysis of some LPA-responsive genes in OVCAR3 cells

Genes	Fold Increase
Cytokines and Angiogenic Factors	
Interleukin 8 (IL-8)	31.5
Interleukin 6 (IL-6)	13.2
GROα oncogene	28.3
Proteases	
Plasminogen Activator, tissue type (tPA)	9.4
Urokinase Plasminogen Activator (uPA)	13.8
Adhesion Molecules	
Fibronectin 1	17.6
Integrin alpha-3 subunit	5.7
Signaling Molecules	
Protein Tyrosine Kinase NET	7.7
MAP Kinase phosphatase (MKP-2)	5.4
Protein tyrosine phosphatase	7.6
V-ski	11.2
Apoptosis	
Forkhead	9.2
Defender against cell death	7.2

1.5 Regulation of Transcriptional Activation

Transcriptional activation represents an important point in the control of gene expression. Many transcription activators are formed by the interaction of several protein subunits some of which directly bind DNA or attach to bound complexes via non-covalent

interactions. Emerging evidence suggests that the influence of LPA on gene expression to a large extent involves activation of transcription factors [67-69]. With 10% of genes in the human genome coding for transcription factors, this group are the single largest family of human proteins (approx. 2600 members) [70, 71]. Transcription factors bind to unexpressed portion of the DNA mainly made up of specific sequences of regulatory modules. These modules contain distal enhancer elements, core or basal promoter elements and proximal promoter elements. The TATA element, located 25 base pair (bp) from the transcription start site and a pyrimidine-rich initiator (Inr) element found at the transcription start site, represent major modules of the core promoter element [72, 73]. Both elements can function independently or synergistically [73]. A cell responds to stimuli such as growth factors and hormones by turning off or on signaling cascades that usually peaks with activation of one or more transcription factors.

Many transcription factors consist of one or more DNA binding domain (DBD) [74]. They also often possess a trans-activating domain (TAD) and/or a signal sensing domain (SSD) [75]. The DBD and TAD domains of a single transcription factor can function independently [74]. Transcription factors positively or negatively modulate the expression of their target genes. Therefore, a transcription factor can be an activator (promoting transcription) or a repressor (downregulating or suppressing transcription). Functionally, a transcription factor can either be constitutively-active (present in the cell all the time) or conditionally-active (requiring cell-specific or external signal for activation).

The most important tumor suppressor gene is protein p53, a transcription factor activated following cellular stress [76, 77]. More than 40% of epithelial ovarian

carcinomas are known to harbor inactivating mutations in p53 gene [78, 79]. The presence of inactivating p53 mutation in many cancer types underscores the importance of the anti-proliferative functions of this transcription factor. In fact, drug resistance in cancer therapy has been associated with p53 mutations [80-82]. Unlike p53, most of the other transcription factors known to play important roles in the proliferation and survival of cancer cells are either overexpressed or highly activated. Several studies have described the general mechanisms for the activation of common transcription factors including activator protein 1 (AP-1), signal transducers and activators of transcription (STATs), specificity protein 1 (Sp-1), CCAAT/enhancer binding proteins (C/EBPs) and nuclear factor-kappa light chain enhancer of B cells (NF-κB). However, specific information of how cellular context might modulate the activities of these proteins in many human malignancies including ovarian cancer is still lacking. Many LPA-target genes harbor binding sites for a common subset of transcription factors in their promoters, suggesting common mechanisms for their regulation by LPA (Fig. 1.2). Targeting pathways that activate these transcription factors remains an attractive option for the treatment of cancer. Mechanistic details of the activation of these transcription factors by LPA in ovarian cancer will promote better understanding of ovarian oncogenesis and may lead to identification of novel targets for treatments of ovarian cancer.

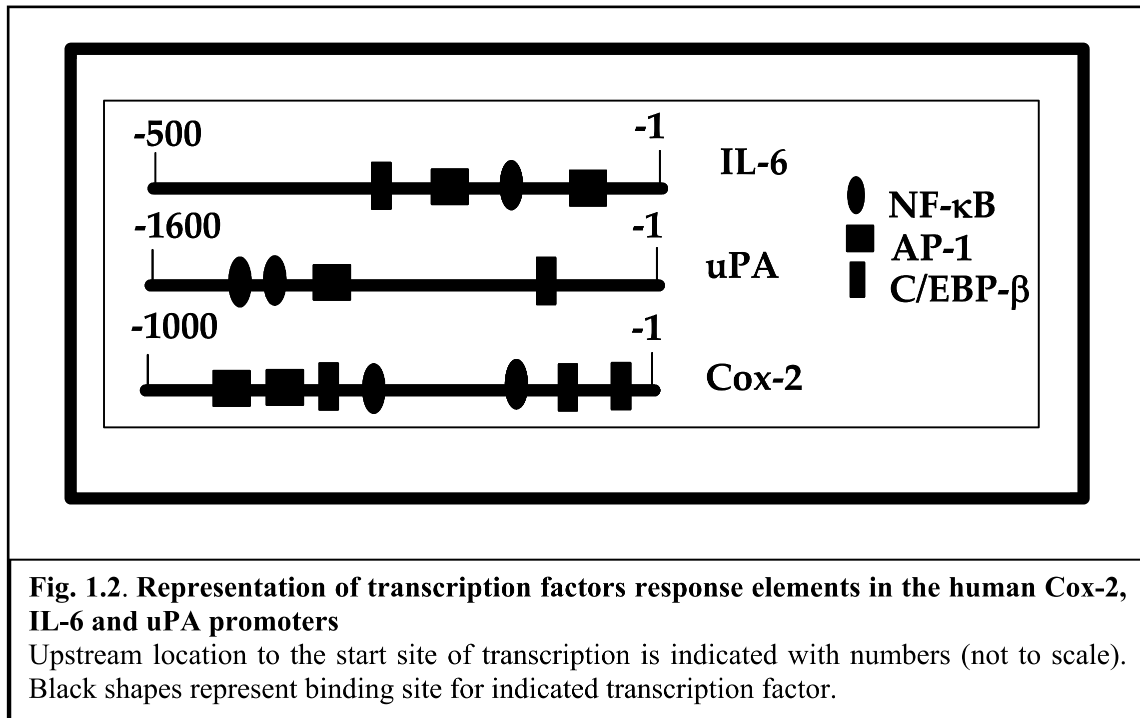


Fig. 1.2. Representation of transcription factors response elements in the human Cox-2, IL-6 and uPA promoters
Upstream location to the start site of transcription is indicated with numbers (not to scale). Black shapes represent binding site for indicated transcription factor.

1.5.1 Activation of AP-1 family of Transcription Factors

AP-1 proteins belong to the bZIP family, a group of protein that possesses a bipartite DNA-binding motif consisting of a basic region for DNA contact and a leucine zipper region for dimerization. AP-1 members include the Jun proteins (c-Jun, Jun B, Jun D), ATF and Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) [83, 84]. These transcription factors play a central role in the regulation of gene expression and cell transformation. AP-1 controls cellular processes such as differentiation, proliferation and apoptosis in response to a variety of stimuli including growth factors, stress, cytokines and bacterial and viral infections [84, 85]. AP-1 proteins interact with a TPA (12-*O*-tetradecanoylphorbol-13-acetate) responsive element (TRE) TGAC/GTCA on the promoter and enhancer regions of

their target genes, and bind DNA after dimerization [86, 87]. Fos proteins though lacking DNA-binding domain, become transcriptionally active when they form heterodimers with other AP-1 components. In contrast, Jun proteins can form both homodimers and heterodimers though Jun-Fos heterodimers are more stable and therefore favored [88]. In fact, the re-introduction of c-Fos in F9 teratocarcinoma cells was shown to enhance the transcriptional and transforming properties of c-Jun and JunB [89, 90]. Dimers of AP-1 proteins can stimulate or repress transcription. While c-Jun/c-Fos heterodimers are known transcriptional activators, Jun B/c-Fos complexes are mostly repressors of transcription [91-93]. Since c-Jun and c-Fos members vary significantly in their relative abundance in different cell types, a complex network of transcriptional regulators is formed when these proteins interact with family members and with additional proteins. AP-1 proteins dimerize efficiently with other transcription factors such as ATF/CREB family of proteins [94-96].

Nearly all AP-1 components have been implicated in tumor development and progression; and many of these proteins have also been shown to possess transforming potentials [97, 98]. The expression pattern of AP-1 proteins in tumors varies depending on tissue type [99-101]. For example, high expression levels of Fra-1 and Fra-2 are associated with metastatic cell lines such as mouse mammary adenocarcinoma CSML-100 [102, 103]. However, no detectable expression of c-Fos or Fos B was found in this cell type. In a closely associated weakly invasive and non-metastatic CSML-10, only c-Fos was detected; but the expression of c-Jun remained essentially the same in both cell lines [102]. Immunohistochemical studies using well differentiated endometrioid endometrial tumor samples showed significant correlation of high tumor grade or disease stage with

expression of c-Fos [101]. The same studies showed that the overexpression of c-Fos seem to substitute for the expression and perhaps the role of Fra-1 in non-endometriod tumors such as breast carcinomas. The role of various AP-1 components in other cancer types is inconclusive. For instance, reduced cell viability was observed in ovarian cancer cells overexpressing a dominant-negative form of c-Fos in the presence of non lethal doses of cisplatin, an anticancer drug [104]. However, another experimental system demonstrated that c-Fos protein levels in ovarian carcinoma cell lines correlate with response to paclitaxel therapy in nude mouse xenograft [105].

In many cell types, expression of AP-1 proteins is often temporally modulated in response to stimuli. As such, the functional activity of AP-1 in a particular cell is not only dynamic with respect to time but also a function of the differentiation state and environment of the cell. The DNA binding activity and transcriptional capacity of AP-1 proteins are greatly affected by post-translational modifications particularly phosphorylation. The c-Jun N-terminal kinases (JNK or stress activated protein kinase SAPK), members of the MAPK family, are the mediators of phosphorylation of Jun family members [106] while a variety of proteins have been reported as putative c-Fos kinases including MEK5, RSK and p38 MAPKs [107-109]. c-Jun is phosphorylated by JNK at specific serine residues. In particular, phosphorylation of c-Jun at Serine 63 and 73 located within its transactivation domain increases its transactivation capacity [106]. Studies show that deacetylation of c-Jun by CBP, a histone deacetylase enhances the transcriptional activity by several folds [110, 111]. Further, the promoter of c-Jun harbors the binding sites of many transcription factors including AP-1 itself. Thus, agonist-induced c-Jun

transcription is often followed by an increase in the expression of c-Jun. This positive auto-regulatory loop is a common phenomenon shared by many other AP-1 components [112-114]. AP-1 target genes include MMPs [115], uPA [116], VEGF [117], CD44 [118], and Bcl-2 [119]. A subset of these genes responds to LPA stimulation in diverse cell types [12, 62].

1.5.2 Activation of C/EBP

The C/EBP family is made up of six members: C/EBP-α, C/EBP-β, C/EBP-γ, C/EBP-δ, C/EBP-ε, and C/EBP-ζ [120, 121]. With the exception of C/EBP-ε, and C/EBP-ζ, this subfamily of transcription factors belong to the exclusive group of liver-enriched transcription factors having been first discovered in the liver. C/EBPs are also members of the bZIP family of proteins [121]. C/EBPs form homo- and heterodimers with family members and with other bZIP family of transcription factors including the AP-1 proteins c-Jun and c-Fos [122, 123]. C/EBPs recognize a specific palindromic sequence in the major groove of DNA. It has been proposed that dimerization between two groups of leucine zipper proteins brings the basic amino acids of DNA binding domain into close proximity [120, 124, 125]. Hence C/EBPs dimerization is a prerequisite for DNA binding and dimers readily dissociate into monomers when not bound to DNA. The C/EBP proteins also contain activation and regulatory domains in the N-termini [125].

C/EBPs play important roles in cell proliferation and differentiation, liver regeneration, energy metabolism, tumorigenesis and other physiological processes [121,

126, 127]. Although tissue expression patterns of C/EBPs often overlap, there exist significant differences in the functions of each member of the family [128]. C/EBP-$\alpha^{-/-}$ mice are neonatal lethal due to hypoglycemia and lack of stored liver glycogen, accentuating the role of C/EBP-α in glucose metabolism and terminal differentiation of adipogenesis and hematopoiesis [124, 129]. However, C/EBP-$\beta^{-/-}$ mice are viable with serious defects in hematopoiesis and immune system [130, 131]. In addition, these mice showed a defective female reproduction system [132]. The loss of fertility in C/EBP-$\beta^{-/-}$ mice underscores the involvement C/EBP-β in ovarian follicular development and corpus leteum formation, enhancing the effects of lutenizing hormone (LH/hCG) [132-134]. Importantly, C/EBP-β is preferentially expressed in endometrial adenocarcinoma and has been shown to be overexpressed in ovarian cancer. Its expression level highly correlates with progression of the disease [135].

In some cell systems, expression of C/EBP-β gene can be induced by inflammatory cytokines, steroid hormones and growth factors [122, 136]. The effects of these stimuli on C/EBP-β expression can be simple in some cellular context but complicated in others. Insulin is a classical modulator of C/EBP-β expression in the liver [127]. In a rat hepatoma cell line, co-stimulation with insulin resulted in attenuation of C/EBP-β mRNA expression induced by cytokines and dexamethasone, while on its own, insulin increased C/EBP-β mRNA [137, 138]. The mechanisms involved in the regulation of C/EBP-β expression in other tissues types are not fully understood.

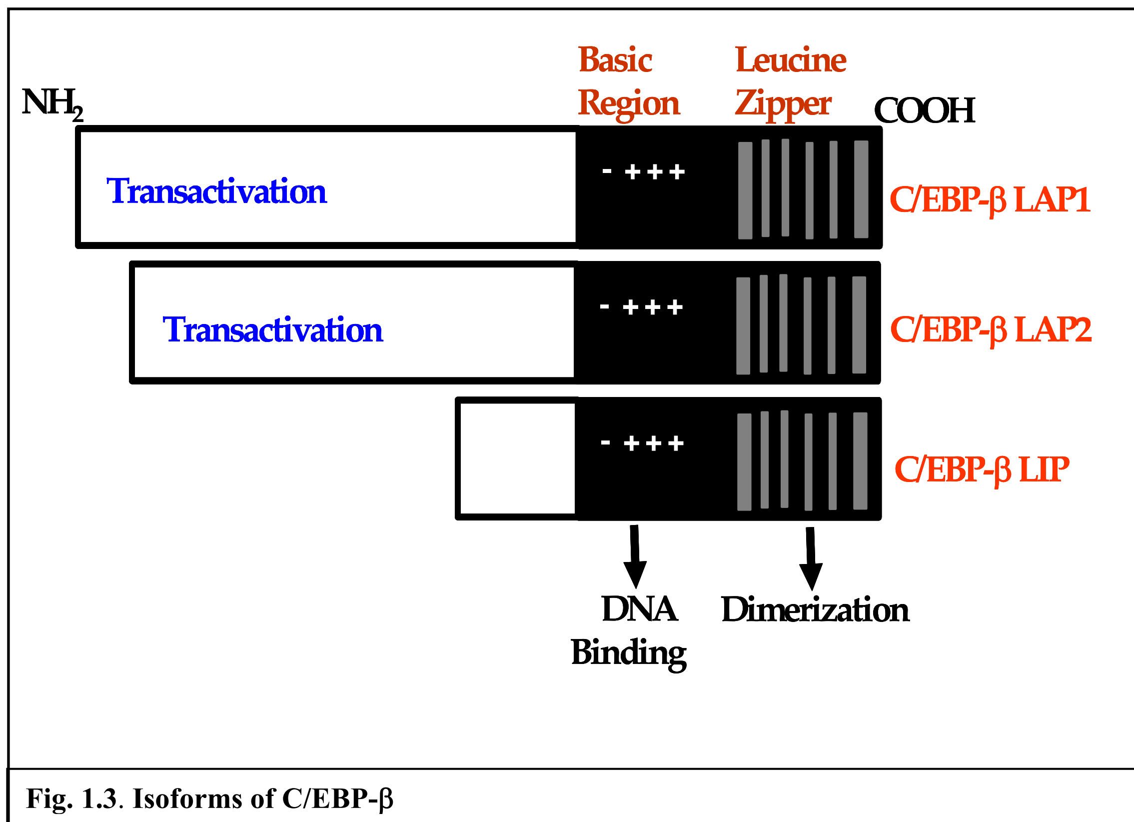


Fig. 1.3. Isoforms of C/EBP-β

There are 3 isoforms of C/EBP-β: liver-enriched activating proteins (LAP1 and LAP2) and liver-enriched inhibiting protein (LIP), a consequence of alternative translation initiation sites within the C/EBP-β mRNA (Fig. 1.2) [138, 139]. LIP lacks a transactivation domain. The dimerization of LIP with LAP leads to inhibition of LAP transcriptional activity; thus LIP functions as a naturally-occurring transcriptional inhibitory or dominant-negative (DN) form of C/EBP-β. Cellular LIP/LAP ratio has been shown to be critical in cell-fate determination [140, 141].

Apart from the differential expression of LAP and LIP, the transcriptional activity of C/EBP-β can be modulated by several other mechanisms including post-translation

modifications, nucleo-cytoplasmic shuttling and direct protein-protein interaction between C/EBP-β and transcription factors of other classes [123, 142]. Phosphorylation of C/EBP-β at specific serine or threonine residues is an important event that often results in the increase in its transcriptional activity [121, 125, 126]. However phosphorylation at other sistes such as Serine 173, 223 and 240 may decrease its DNA binding activity [121,123, 142]. The kinases that mediate the phosphorylation of C/EBP-β include PKA, PKC, Camodulin Kinase II and MAP kinase [125]. These kinases lie downstream of diverse signaling cascades including those of GPCRs.

C/EBP-β can interact with other transcription factors including Sp1, AP-1 and NF-κB to activate transcription. This feature is mediated by the leucine zipper and the DNA binding domains [123, 143]. Heterodimeriztion between C/EBP and other transcription factors could enhance transcription activity of individual dimer partners. The crosstalk could also bring about changes in target specificity. For example, CREB/ATF and C/EBP-β heterodimer causes C/EBP-β to bind onto palindromic cAMP responsive elements (CREs) on the DNA rather than the CCAAT C/EBP binding sites resulting in regulation of different target genes [144]. The activation and involvement of C/EBP-β in LPA-induced gene regulation is the focus of work described in Chapter 3 of this dissertation.

1.5.3 Activation of NF-κB

NF-κB is a ubiquitous transcription factor that plays important roles in many physiological and pathological processes. It is a central mediator of several inflammatory responses and immune function. NF-κB is activated by a wide variety of stimuli including inflammatory cytokines (e.g. tumor necrosis factor TNF) and microbial pathogens (e.g. lipopolysaccharide LPS) that bind cell surface receptors [145, 146]. Other important activators of NF-κB are genotoxic stress, DNA damage, UV light, oxidative stress, chemotherapeutic drugs, phorbol esters, growth factors and physiologic mediators such as angiotensin II and PAF [145]. Abnormal regulation of NF-κB has been linked to several disease conditions including inflammatory and autoimmune diseases, septic shock, improper immune development, viral infection and cancer [147, 148].

In vertebrates, the NF-κB family consists of five Rel protein subunits, so called because they all share a common N-terminal Rel homology domain (RHD). Rel proteins may be classified into two groups. The first group consists of RelA (p65), RelB and c-Rel [149]. These subunits possess within their structures a C-terminal transactivation domain (TAD) to promote transcription. The second group, p50 and p52 are synthesized from large precursor molecules p105/p50 (NF-κB1) and p100/p52 (NF-κB2) respectively and lack the TAD [149]. However, p105 and p100 contain a series of five to seven ankyrin repeats that blocks a nuclear localization signal within the RHD. Both of these precursor proteins are processed by cleavage to generate the mature transcription factors, p50 and p52.

Rel proteins form homo- and heterodimers by employing a C-terminal Ig-like domain of about 100 amino acids within the RHD commonly called the dimerization domain (DimD). Twelve of the fifteen possible dimers are able to bind to a consensus sequence (5'-GGGACTTTC-3') on DNA and effectively participate in gene transcription [150-152]. While some Rel dimers, such as RelA/p50 heterodimers are well known as transcriptional activators, some others particularly dimers lacking RelA, RelB or c-Rel such as a p50 and p52 homodimers are generally repressors of κB site transcription [151, 153, 154]. In addition to nuclear localization, dimerization and DNA binding, the RHD also mediates the interaction with the inhibitors of NF-κB, the IκBs. These proteins, sometimes regarded in literature as inhibitory subunits of NF-κB, possess a similar ankyrin repeat domain as in p100 and p105. They however lack the RHD. IκBs include IκBα, IκBβ, IκBγ (derived from c-terminal domain of p100), IκBε IkBζ, Bcl-3, pp40 and avian fever viral protein p28.2 [155-157]. In unstimulated conditions, NF-κB is complexed with IκBs through the ankyrin repeat domain resulting in its sequestration in the cytoplasm (IκBζ is known to retain NF-κB in the nucleus), away from its target genes.

The importance of NF-κB in processes that require rapidly-acting primary transcription factors ("first responders") such as inflammation is underscored by the fact that NF-κB activation does not require new protein synthesis [158]. There are two known pathways for activation of NF-κB in stimulated cells. In the classical or canonical pathway, activating signal induces the degradation of IκB proteins. A typical activator of this pathway is TNFα. Upon ligand-induced TNF receptor activation, multiple signals near

the cell membrane converge at the IκB kinase (IKK) complex that consists of IKKα, IKKβ and IKKγ (or NEMO- "NF-κB essential modulator"). The IKK complex is activated by the interaction of NEMO with a Lys-63-linked polyubiquitinylated receptor-interacting protein 1, RIP1, a serine-threonine kinase. Activated IKKβ phosphorylates IκBα at two serine residues, Serine 32 and Serine 36. This tags the NF-κB inhibitor for multiple ubiquitinylation with subsequent degradation by the 26S proteasome [159]. Previously concealed NF-κB nuclear localization signal becomes exposed targeting the dimer, usually p65/p50 to the nucleus for transcription. Interestingly, IκB itself is one of NF-κB target genes and its expression is upregulated following NF-κB activation.

The alternate or non-canonical pathway of NF-κB activation involves IKKα rather than IKKβ of the IKK complex. This pathway is based on the processing of p100 following cellular stimulation by cytokines such as lymphotoxin β (LT-β), B cell activating factor (BAFF), CD40 ligand and viruses including the Epstein-Barr virus (EBV) [160]. Signal-induced post-translational stabilization of NF-κB inducing kinase (NIK) causes the protein to interact with a homodimer of IKKα thereby activating the latter. A key point in this pathway is IKKα phosphorylation of p100, an event that leads to its polyubitinylation and proteasomal degradation of the C-terminus of the protein. The remnant portion, p52 continues to interact with RelB, now as a transcriptionally-active heterodimer that quickly moves into the nucleus. Beside stimulus-specific activation of either the classical or alternate pathway, a dynamic interaction of different NF-κB dimers

with specific gene promoters provides a critical control of the expression of NF-κB target genes [152].

In addition to IκB-dependent activation the NF-κB, diverse post-translational modifications serve as alternatives to regulate NF-κB activity. p50, an important partner of the most-studied NF-κB dimer RelA/p50, is regulated through processing of its precursor, p105. Similar to p100, stimulus-induced phosphorylation of p105 results in its polyubiquitinylation and subsequent proteolytic degradation to p50. The DNA binding activity of p50 has also been shown to be enhanced by phosphorylation of Serine 337 located within its RHD domain [161]. p65 (RelA) can be phosphorylated by several protein kinases at specific residues and this inducible phosphorylation of p65 is often used as readout of NF-κB activation. A well-studied phosphorylation site is Serine 536 catalyzed by IKKα/β, IKKε or TBK1 (TANK binding kinase 1) [162-164]. Although they share sequence homology with IKKα/β, IKKε and TBK1 are not part of the IKK complex. Phosphorylation of p65 at Serine 536 impairs its interaction with IκBα and increases its nuclear accumulation. However, whether Serine 536 phosphorylation of p65 is required for transcription remains controversial [165-167]. Other site-specific phosphorylation of p65 at serine residues includes Serine 435 (by camodulin kinase IV, CaMKIV), Serine 468 (by IKKβ, IKKε and GSK-3β) [168] and Serine 276 (by catalytic subunit protein kinase A, PKAc) [169]. While phosphorylation of p65 at these residues are known to increase its transactivation potential, phosphorylation of threonine residues have been shown to suppress the activity of NF-κB. p65 phosphorylation of C-terminal Threonine 435 and

Threonine 505 is induced by ARF tumor suppressor, p14ARF in a p53-independent manner [170, 171]. Threonine 505 phosphorylation causes p65 to interact with histone deactelylase 1 (HDAC1), which greatly inhibits p65 transactivation. The phosphoryation of p65 is often the prerequisite for other post-translation modifications such as ubiquitinylation and acetylation that regulate the activity of the protein [172, 173]. Acetylation, like most phosphorylation events often results in enhanced activity of NF-κB [174].

1.6 Crosstalk between GPCRs and Receptor Tyrosine Kinases (RTKs)

Many transcription factors including those described in the preceding sections are substrate for molecules downstream of GPCR-induced signaling cascade, particularly kinases. For example, G_q-dependent activation of IKK with subsequent activation of NF-κB has been described in many systems [175, 176]; and ATF-2 a member of AP-1 family is a substrate for p38/MAPK and the JNK/SAPKs, downstream effectors of MAPK [177]. The stimulation of MAPK pathway via pertussis-toxin sensitive G_i represents a major signaling event downstream of LPA GPCRs. G_i-mediated activation of Ras seems to occur through a tyrosine kinase (TK)-dependent manner [178, 179]. The intracellular TK linking G_i signal to Ras activation has not been identified. Some studies suggest receptor tyrosine kinase (RTK), particularly epidermal growth factor receptor (EGFR), could serve the role [180, 181]. Indeed, many biological functions of GPCRs are known to depend on EGFR [181, 182]. Furthermore, emerging evidences suggest that some ligand-induced RTK

signaling may also require the cooperation of GPCRs [183]. The current dogma for the crosstalk between GPCRs and RTKs suggests that GPCR ligands such as LPA activate cellular responses through transactivation of EGFR or other highly expressed RTK [184]. The so-called transactivation model is not consistent with LPA stimulation of EGFR phosphorylation and activation or with LPA induction of proteolytic release of EGFR ligands such as EGF or HB-EGF in certain cellular systems [180, 185, 186]. However, recent evidence suggests that these two receptor types independently control different pathways leading to Ras activation in response to LPA. Background EGFR activity is necessary for basal nucleotide exchange on Ras, whereas the LPA receptor controls an inducible exchange activity [187, 188]. Thus, activation of Ras by LPA involves two parallel inputs: one directly from GPCR and the other signal from basal EGFR, a mode of action differing from the transactivation model [187, 189].

The work described in Chapters 3 and 4 took advantage of LPA-induced activation of transcription factors as readout to analyze the role of RTKs in LPA regulation of gene expression. Our results indicate that LPA-induced activation of AP-1 and C/EBP-β requires an input from EGFR while activation of NF-kB by LPA is independent of EGFR activity. The differential requirement of EGFR for activation of different transcription factors are underlied by EGFR-dependent or independent G proteins signaling cascades involved in activation of these transcription factors.

CHAPTER 2

REGULATION OF CYCLOOXYGENASE-2 EXPRESSION BY LPA: A PARADIGM OF LPA-INDUCED GENE EXPRESSION

Part of the work presented in this chapter has been published in FASEB Journal 22: 2639-2651 (2008).

2.0 Abstract

Cyclooxygenase-2 (Cox-2) is a key enzyme in the biosynthesis of prostaglandin (PGE) and thus functions as a critical mediator of inflammation. In addition to this well-established role, Cox-2 is implicated in the pathogenesis of human malignancies including colon, breast and skin cancers. The role of Cox-2 and the mechanism for its regulation in ovarian cancer are poorly understood. In the current study, we demonstrated that LPA, a previously identified lipid mediator of ovarian cancer, induced expression of Cox-2 in ovarian cancer cell lines. Treatment of cells with LPA resulted in a rapid and robust accumulation of PGE_2 in culture supernatants, indicating that LPA-induced Cox-2 expression leads to PGE_2 synthesis and release. We downregulated LPA receptors expression with siRNA and found that only a subset of LPA receptors participate in LPA-induced Cox-2 expression. The effect of LPA involves both transcriptional activation and post-transcriptional enhancement of Cox-2 mRNA stability. The consensus sites for C/EBP

in the Cox-2 promoter were essential for transcriptional activation of Cox-2 by LPA. The NF-κB and AP-1 transcription factors commonly involved in inducible Cox-2 expression were dispensable. Dominant negative form C/EPB-β inhibited LPA-induced activation of the Cox-2 promoter and expression. The RNA stabilization protein HuR bound to and protected Cox-2 mRNA in LPA-stimulated cells, indicating an active role for HuR in sustaining Cox-2 induction during physiological responses to LPA

2.1 Introduction

LPA is a naturally occurring phospholipid mediator of diverse biological activities [3, 7, 29, 190]. It is produced by activated platelets during coagulation and thus is a normal constituent of serum [14]. At least seven G protein-coupled receptors (GPCRs) of LPA have been identified. The LPA_1/Edg2, LPA_2/Edg4 and LPA_3/Edg7 receptors are members of the endothelial cell differentiation gene (Edg) family and share 50-57% homology in their amino acid sequences [36-38]. LPA_4/$P2Y_9$/GPR23 and LPA_5/P_2Y_5 of the P2Y family of receptors are two novel LPA receptors structurally distant from the LPA receptors of the Edg family, sharing only 20-24% homology with LPA_{1-3} [39, 40]. More recently, LPA has also been identified as a possible ligand for two additional orphan receptors GPR87 and P2Y10 [41, 42]. In addition to these cell surface GPCRs, LPA also been shown to bind and activate the peroxisome proliferator–activated receptor γ (PPARγ) which plays critical roles in controlling fat and energy metabolism [55].

A number of G-protein-dependent signaling cascades have been identified as potentially mediating the actions of LPA e.g. stimulation of phospholipases C and D [16, 49], inhibition of adenylate cyclase [49], activation of Ras and the downstream mitogen-activated protein kinase (MAPK), and tyrosine phosphorylation of focal-adhesion proteins [50, 52]. Activation of these signaling events downstream of LPA receptors culminates in cell morphological changes, cell growth, survival and migration [50, 51]. Recently, we and others described that LPA is also a potent modulator of gene expression, in particular, the genes involved in the inflammatory processes and carcinogenesis [9, 62, 191-195]. The effect of LPA on gene expression has been mainly investigated in human ovarian cancer cells wherein both LPA receptors (LPA_2 and LPA_3) and LPA levels are found to be upregulated [4, 62]. A number of inflammatory cytokines, angiogenic factors and oncoproteins such as interleukin-6 (IL-6) [9, 191], interleukin 8 (IL-8) [9], vascular endothelial growth factor (VEGF) [193], urokinase plasminogen activator (uPA) [194] and cyclooxygenase-2 (Cox-2) [195] have been reported to be induced induced by treatment of ovarian cancer cells with LPA .

Cyclooxygenases are involved in biosynthesis of prostaglandins (PGE) from arachidonic acid (AA) [196]. Cox-1 is constitutively expressed in most cell types while Cox-2 is an inducible form, upregulated by pro-inflammatory cytokines, stress and growth factors [196]. In addition to the well established role in inflammation, Cox-2 has been implicated in human carcinogenesis, particularly in cancers of the colon, breast and skin [196-198]. Pharmacological suppression of Cox-2 activity with specific inhibitors reduces the number and size of adenomas in patients with familial adenomatous polyposis and

prevents colon cancer development [196-198]. The role of Cox-2 in the development of other types of malignancies including ovarian cancer is more controversial. Recent evidence indicates that a majority of ovarian tumors including serous, endometroid, clear cell and mucinous carcinomas and borderline tumors display positive Cox-2 immunoreactivity with approximately 70% overall cases showing moderate to high levels of expression [195]. LPA, a lipid mediator present in ascites of ovarian cancer patients [62], is a potent stimulus of Cox-2 expression in ovarian cancer cell lines [195]. Because both expression of LPA receptors and LPA levels are elevated in ovarian cancer [62], the ability of LPA to induce Cox-2 gene expression may reflect a physiological role for LPA in regulation of prostaglandins in ovarian tumor cells *in vivo*. In addition, genetic deletion of the LPA_3 receptor in mice leads to a delayed implantation and defective embryo spacing, associated with reduced uterine expression of Cox-2 mRNA in the LPA_3-deficient female mice [199], suggesting that LPA is an endogenous regulator of prostaglandin generation in the uterus crucial to mammalian reproduction.

Despite the prominent role of LPA signaling in regulation of Cox-2 [195, 199, 200], little is known about the LPA receptors, intracellular signaling pathways and transcription factors involved in the process. The results presented in the current work demonstrate that LPA-induced expression of Cox-2 involves both transcriptional and posttranscriptional regulation. The transcriptional activation of Cox-2 by LPA is mediated primarily by the CCAAT enhancer-binding protein (C/EBP) transcription factor independently of other transcription factors such as NF-κB and AP-1 commonly involved in inducible Cox-2 expression. Further, we demonstrated that the transcriptional

stimulation is reinforced by posttranscriptional protection of Cox-2 mRNA stability mediated by the RNA binding protein HuR, leading to sustained induction of Cox-2 in LPA-treated cells.

2.2 Materials and Methods

Materials 1-Oleoly (18:1) LPA and sphingosine 1 phosphate (S1P) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Prior to use, these phospholipids were dissolved in PBS containing 0.5% fatty acid-free bovine serum albumin (BSA). BSA, Fugene 6 and protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). The PGE_2 EIA kit, NS-398 and AA were purchased from Cayman Co. (Ann Arbor, MI). [^{3}H]-AA and [^{32}P]-dCTP were purchased from Perkin Elmer (Boston, MA) and Amersham Biosciences (Piscataway, NJ), respectively. Plasmid DNA was purified using the endo-free purification kit from Qiagen (Valencia, CA). Luciferase assay reagents were obtained from Promega (Madison, WI). GW9662, and pharmacological inhibitors of MAPKs were from Calbiochem (San Diego, CA). Pertussis toxin (PTX) was purchased from List Biological Laboratories, Inc. (Campbell, CA). All oligonucleotides and primers were synthesized by Operon Biotechnologies, Inc (Huntsville, AL). Phospho-specific antibodies for phosphorylated ERK, JNK, CEBP-β and anti-tubulin α/β antibodies were obtained from Cell Signaling (Danvers, MA). The monoclonal antibodies against Cox-2 and HuR and a polyclonal antibody against Cox-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Insulin, TRIzol and cell culture medium were obtained from Invitrogen Inc.

(Carlsbad, CA). Bovine fetal serum was from Biomeda (Foster City, CA). Insulin-like growth factor I (IGF-I) was from Upstate Biotechnology (Lake Placid, NY). Hepatocyte growth factor (HGF) was from R & D systems (Minneapolis, MN). Epidermal growth factor (EGF), AG1478 and anti-β-actin monoclonal antibody were obtained from Sigma-Aldrich (St. Louis, MO).

Plasmids The C/EBP-β, liver-enriched transcriptional activator protein 1 (LAP1) and LAP2 expression vectors were kindly provided by Dr. L. Sealy (Vanderbilt University School of Medicine) [139, 201]. The expression of C/EBP-β from these vectors in transfected cells was confirmed by immunoblotting. The dominant negative form of C/EBP-β, LIP (liver-enriched inhibitory protein), [202] was cloned into pcDNA3.1 by RT-PCR amplification of a 444 bp cDNA fragment of C/EBP-β from Caov-3 cells (see primer details in Table 2.1). The structure of pcDNA3-LIP was confirmed by automatic sequencing and immunoblotting analysis of expression of the short, truncated form of C/EBP-β (21 kD) [201] in transfected cells.

Cell Culture The sources of ovarian cancer cell lines used in the study were described previously [9, 192]. These cells were cultured in RPMI medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. All cell lines were frozen at early passages and used for less than 10 weeks in continuous culture.

Western Blot Cells were lysed in SDS sample buffer or in ice-cold X-100 lysis buffer [1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 100 mM NaF, 10 mM Na PPi, and protease inhibitor cocktail]. Total cellular proteins were resolved by SDSPAGE, transferred to Immun-Blot membrane [poly(vinylidene difluoride)] (BIO-RAD, Hercules, CA), and immunoblotted with antibodies following the protocols of manufacturers. Immunocomplexes were visualized with an enhanced chemiluminescence detection kit from Amersham (Piscataway, NJ) using the horseradish peroxidase–conjugated secondary antibodies (Cell Signaling, Danvers, MA).

Quantitative determination of PGE_2 in culture supernatants Ovarian cancer cell lines were plated in 6-well plates and grown to 60% confluence in complete medium. The cells were starved for 24 hours before stimulation with LPA or vehicle for the specified periods of time. The levels of prostaglandins E2 (PGE_2) present in culture supernatants were quantified using the PGE_2 EIA kit.

AA release Ovarian cancer cell lines were plated in 6-well plates and grown to 60% confluence in complete medium. The cells were labeled with 1 μCi [^{3}H] AA/well in 2 ml of serum-free DMEM for 20 hours. The cells were washed 3 times with DMEM and incubated with DMEM containing 0.1% fatty acid-free BSA (DMEM+BSA) for 3 hours. The cells were then refed new DMEM+BSA and incubated with LPA or ATP for the indicated periods of time. The cells were dissolved in 2 ml of 0.2 N NaOH overnight. The

radioactivity in the supernatants and cells was determined by scintillation spectrometry. The AA release was presented as percentages of the activity present in medium versus the total labeling in both medium and cells.

Northern blot and mRNA stability assays Total cellular RNA was extracted from cell lines using the TRIzol reagent following the instruction of the supplier. RNA samples were electrophoresed on agarose gel containing formaldehyde, stained with ethidium bromide, and transferred to N^+ hybrid nylon. RNA was immobilized with UV cross-linking, prehybridized and hybridized to ^{32}P-labeled cDNA probes at 65^oC overnight in a hybridization buffer (1% BSA, 0.5 M NaH_2PO_4, 1 mM EDTA, 7% SDS, 10 μg/ml salmon sperm DNA). The cDNA of the human Cox-2 and LPA receptors genes were isolated by RT-PCR amplification from Caov-3 cells. The ^{32}P-deoxy-CTP-labeled DNA probes were prepared using the High Prime labeling system (Roche). Equal loading of RNA samples was confirmed by rehybridization to the cDNA of 18S rRNA (ATCC). To determine Cox-2 mRNA stability, Caov-3 cells were treated with or without 10 μM LPA for 6 hours before actinomycin D (5 μg/ml) was added to stop new RNA synthesis. Total cellular RNA was isolated from the cells using TRIzol at 0, 2, 4 and 6 hr after addition of actinomycin D. Reverse transcription was performed to synthesize single stranded cDNAs using ThermoScript (Invitrigen). The relative levels of Cox-2 mRNA were quantified by qPCR using the human Cox-2 specific probe and the TaqMan system from Applied Biosystems (Foster City, CA).

siRNA - The human LPA_{1-3} and LPA_5 receptor SMARTpool siRNAs and the Bcl10 siRNAs and the non-target control siRNA (see Table 2.1) were purchased from Ambion (Austin, TX). The specific siRNA or non-target control siRNA (2.25 μg) was transfected into ovarian cancer cell lines (1.25 $x10^6$ cells) with Amaxa nucleofector II (Kit T, Program T32). The transfected cells were cultured in 6-well plates in complete medium. After 48 hours, the cells were starved in serum-free RPMI 1640 and stimulated with LPA for 6 or 12 hours for Cox-2 induction. RNA was isolated from parallel cultures for RT-qPCR analysis to determine the efficiency of siRNA knockdown.

The reporter vectors and luciferase assays The proximal sequence (–980 to +15) of the human Cox-2 gene promoter [202] was cloned from the genomic DNA of Caov-3 cells by PCR amplification and inserted into the pGL2-Basic vector (Promega) and verified by automatic sequencing. The 7.2 kb Cox-2 promoter fragment was kindly provided by Dr. TM McIntyre (Cleveland Clinic Foundation) [202] and were cloned into the pGL2-Basic vector. Ovarian cancer cell lines were seeded in 6-well plates and grown to 30-40 % confluence before transfection with the luciferase vectors using Fugene 6 (Roche) or TransIT-TKO (Mirus Bio Corp., Madison, WI) according to the instructions of the manufacturers. About 48 hours after transfection, the cells were starved for 24-36 hours before stimulation with LPA or vehicle for 6 hours. Cell extracts were prepared and assayed for luciferase activity using the luciferase assay kit from Promega. The luciferase activity was normalized on the basis of the activity of cotransfected β-galactosidase reporter driven by the cytomegalovirus promoter (pCMVβ-gal).

Table 2.1 Plasmids and Primers

Target Gene	Primers
Cox-2-1kb-Luc	5'-TTTAGCGTCCCTGCAAATTCTGGC-3'(SENSE) 5'-CGCTCACTGCAAGTCGTATGACAA-3' (ANTISENSE).
Cox-2 cDNA	5'-AGATCATAAGCGAGGGCCAGCTTT-3' (SENSE) 5'-ACTTTCTGTACTGCGGGTGGAACA-3' (ANTISENSE).
LPA_1 cdna	5'-TGGTGGTCATTGTGGTCATCTGGA-3'(SENSE) 5'-AAGGTGGCGCTCATTTCTTTGTCG-3' (ANTISENSE).
LPA_2 cdna	5'-TACAACGAGACCATCGGCTTCTTC-3'(SENSE) 5'-GCAAGAGTACACAGCATTGAC-3' (ANTISENSE).
LPA_3 cdna	5'-AATTGCCTCAACATCTCTGCC-3'(SENSE) 5'-TATGTACTGGCTGCCTGTGTCACT-3' (ANTISENSE).
LPA_4 cdna	5'-CGCCACCATGGACTACAAG-3'(SENSE) 5'-AAGAGGCTGAAATACCGCCACTGA-3' (ANTISENSE).
LPA_5 cdna	5'-CAGAGCAACACGGAGCACAGGT-3'(SENSE) 5'-GATGCAGCTGCCGTACATGTTCATCT-3' (ANTISENSE).
LIP	5'-GGAATCAAGCTTGGCGCACATGGCGGCG-3' (SENSE) 5'-GCAATACTCGAGCGCTAGCAGTGGCCGGAGG-3' (ANTISENSE).
GAPDH	5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'(SENSE) 5'-CATGTGGGCCATGAGGTCCACCAC-3' (ANTISENSE).
Cox-2 3' UTR-T7	5'-TCCTAATACGACTCACTATAGGGAAGTCTAATGATCATATTTAT-3' (SENSE) 5'-GCTATTTAGGTGACACTATAATCATGGAAGATGCATTG-3' (ANTISENSE).
Cox-2 484 cdna fragment	5'-TGTTCCACCCGCAGTA-CAGAAAGT-3' (SENSE) 5'-GCGGGAAGAACTTGCATTGATGGT-3' (ANTISENSE).
HuR siRNA	5'- GGAUGAGUUACGAAGCCUGtt -3' (SENSE) 5'- CAGGCUUCGUAACUCAUCCtg -3' (ANTISENSE).
Bcl10 siRNA1	5'- GGAAAACCCAAAAGGUCUGtt -3' (SENSE) 5'- CAGACCUUUUGGGUUUUCCtg -3' (ANTISENSE).
Bcl10 siRNA2	5'- GGUCUGGACACCCUUGUUGtt -3' (SENSE) 5'- CAACAAGGGUGUCCAGACCtt -3' (ANTISENSE).
Bcl10 siRNA3	5'- GCAUACUUCUAGGAUAGCUtt -3' (SENSE) 5'- AGCUAUCCUAGAAGUAUGCtt -3' (ANTISENSE).
Non-target control siRNA	5'- AGUACUGCUUACGAUACGGtt -3' (SENSE) 5'- CCGUAUCGUAAGCAGUACUtt -3' (ANTISENSE).

Chromatin Immunoprecipation (ChIP) Assay - Cells were fixed by cross-linking the chromatin with 1% formaldehyde for 5 min, cells and then were scraped with a rubber policeman and collected by centrifugation. The cells were lysed gently with a hypotonic buffer [10 mM Tris-HCl (pH 7.4), 10 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40], and then sonicated six times, 15 s each with 1-min intervals on ice by using a Sonic Dismembrator (Fisher Scientific). The average fragment size was 600 bp under these conditions. An equal amount of chromatin was incubated with at least 5 μg of either C/EBP-β-specific immunoglobulin G (IgG) or normal mouse IgG (Santa Cruz Biotech) at 4°C overnight. IP products were collected after incubation with protein G-coated sepharose beads (Amersham). The beads were washed and protein-DNA complexes were eluted and then cross-links were reversed by incubating at 65°C in the presence of 0.2M NaCl. After digestion of proteins with Proteinase K (0.5 μg/ml) at 55°C for 1 hour, DNA was purified by Qiagen PCR purification columns according to manufacturer's instruction. Purified DNA from the input and IP samples were subjected to PCR with Cox-2 promoter specific primers and analyzed by agarose gel electrophoresis.

Deletion and site-directed mutagenesis - The unique AP-1-like site at around –577 was deleted from the pGL2-Cox2-1kb-Luc by restriction digestion with Af1III followed by re-circulation of the plasmid. The consensus sequences of NF-κB and C/EBP transcription factors present within –980-+15 of the Cox-2 promoter in pGL2-Cox2-1kb-Luc were mutated into inactive sequences using site-directed mutagenesis kits from Stratagene (Cedar Creek, TX). The distal NF-κB site GGGGATTCCCTG was changed to

CGTCATTCCCTG and the proximal NF-κB site GGGGACTACCCC mutated to GGTCACTACCCC. The distal C/EBP site GCCTTTCTTAAC was mutated to GCCCCTATTAAC and the proximal one GGCTTACGCAAT converted to GACTTACGCTCT. The desired deletion and mutation of these binding sites for AP-1, NF-κB and C/EBP were confirmed by automatic sequencing before the plasmids were used for luciferase assays.

Binding of Cox-2 transcripts with HuR *in vitro* and *in vivo* To assess the interaction of HuR with Cox-2 mRNA, the 3' un-translated region (3'-UTR) of Cox-2 mRNA (375 bp from nt 1950-2325, NM_000963) was reversely transcribed and PCR amplified from Caov-3 cells using primers containing the T7 promoter sequences (Table 2.1). The PCR product was purified and utilized as a template for *in vitro* transcription. The transcripts equivalent to the 3'-UTR of Cox-2 mRNA was synthesized and labeled with biotin-11-CTP by transcription from the T7 promoter using *in vitro* transcription kit (Promega). Lysates from control and LPA-treated cells were incubated with the biotinylated Cox-2 3'-UTR transcripts in 1X binding buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 250 mM NaCl, 0.5% Triton X-100] for 30 min at room temperature. The binding complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Invitrogen) and washed thoroughly with PBS followed by Western blotting analysis of HuR. To assess the association of HuR with Cox-2 mRNA endogenously, Caov-3 cells treated with or without LPA were harvested in PBS by scraping from dishes, pelleted and resuspended in polysome lysis buffer containing 100 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes (pH 7.0),

0.5% NP-40, 1 mM DTT, 100 U/ml RNaseOUT (Promega), and the complete protease inhibitor cocktail (Roche). For immunoprecipitation, 1,500 μg cellular proteins were diluted with 700 μl NT2 buffer [50 mM Tris (pH 7.4), 150 mM NaCl, and 1 mM $MgCl_2$, 0.05% NP-40, 5% fatty acid-free BSA, 1 mM DTT, 200 units/ml RNaseOUT and 15 mM EDTA] and incubated for 2 hours with 7.5 μg anti-HuR antibody or an IgG1 isotype control antibody. The immunocomplex was incubated for 1 hour with protein A Sepharose beads (GE Biosciences), and washed thoroughly with ice-cold NT2 buffer. After digestion of proteins present in the beads with Proteinase K (0.5 mg/ml) at 55°C for 20 min, the bead-free supernatants were extracted with phenol/chloroform and precipitated with 0.3 M NaAc, 150 μg/ml glycogen and 2.5 volumes of 100% ethanol. The precipitates were dissolved in 15 μl of nuclease-free water. Potential contamination with genomic DNA was removed using the DNA-free™ DNase Treatment & Removal kit (Ambion). Reverse transcription was performed on 5 μl of the samples with the ThermoScript kit (Invitrogen) followed by PCR amplification of a fragment of 481 bp close to the 3' UTR of Cox-2 mRNA.

Statistics All numerical results were presented as mean ± SD. The statistical significance of differences was analyzed using Student's *t*-test, where $P<0.05$ was considered statistically significant.

2.3 Results

2.3.1 LPA induces Cox-2 protein expression in ovarian cancer cells

Several studies have shown that LPA induces expression of Cox-2 in various cell types including renal mesangial cells and colon and ovarian cancer cells [195, 200, 203]. However, the molecular mechanism regulating Cox-2 gene expression in response to LPA is poorly understood. LPA induced robust and sustained expression of Cox-2 protein in several ovarian cancer cell lines examined, with the most striking effect seen in Caov-3 and OVCA-429 cells (Fig. 2.1).

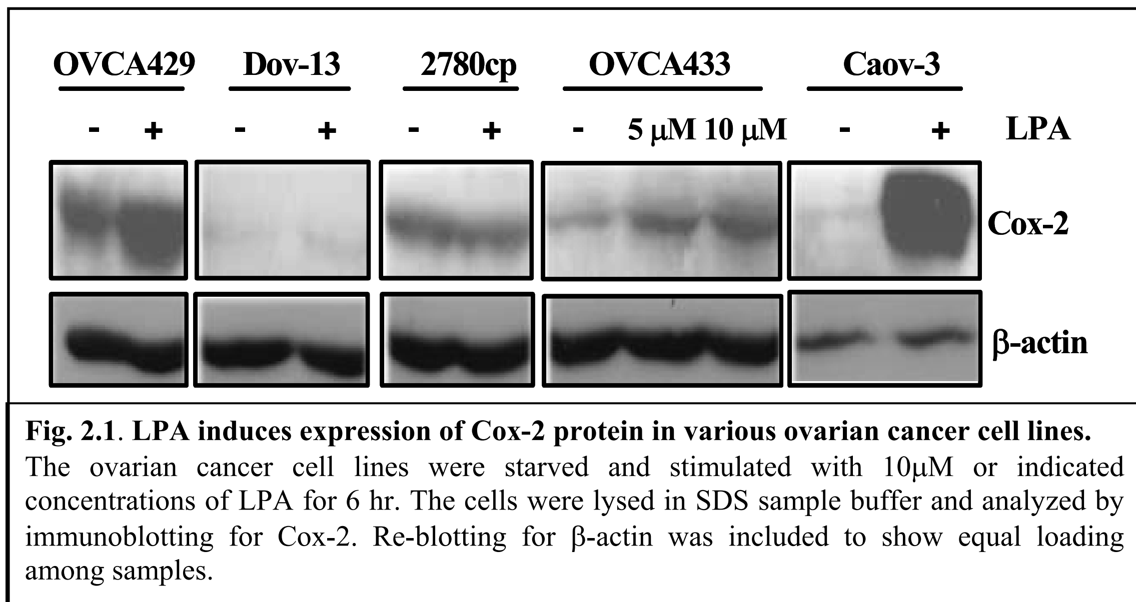


Fig. 2.1. **LPA induces expression of Cox-2 protein in various ovarian cancer cell lines.** The ovarian cancer cell lines were starved and stimulated with 10μM or indicated concentrations of LPA for 6 hr. The cells were lysed in SDS sample buffer and analyzed by immunoblotting for Cox-2. Re-blotting for β-actin was included to show equal loading among samples.

We chose the most responsive Caov-3 and OVCA-429 cells for further characterization. In both cell lines, the effect of LPA was detectable at sub-micromolar concentrations and the maximum effect was achieved with 1-10 µM of LPA (Fig. 2.2 A and B). Upon treatment with LPA, Cox-2 levels increased significantly within 1 hour and peaked at 12 hours (Fig. 2.2 A and B). LPA did not affect Cox-1 expression at early hour points. We observed a slight increase in Cox-1 in Caov-3 cells only after prolonged incubation with LPA for 12-18 hours (Fig. 2.2 A), likely reflecting an indirect effect of LPA. When Caov-3 cells were cultured in complete medium without starvation, LPA remained capable of stimulating Cox-2 expression (Fig. 2.2 C). However, the basal expression of Cox-2 was higher in unstarved cells and the LPA-mediated induction was weaker compared to that achieved in serum-starved cells (Fig. 2.2 C).

We next compared the effects of LPA, FBS, S1P and the peptide growth factors EGF, HGF, and IGF-1 on Cox-2 expression in Caov-3 cells (Fig. 2.2 D). FBS was able to stimulate a prominent increase in Cox-2 protein. LPA is a component of FBS [14, 62, 190] and may account for the ability of FBS to drive Cox-2 expression. Interestingly, EGF, HGF and S1P only weakly stimulated Cox-2 expression (Fig. 2.2 D). The poor response to S1P suggests that induction of Cox-2 is specifically linked to certain GPCRs and is not a general outcome of GPCR activation.

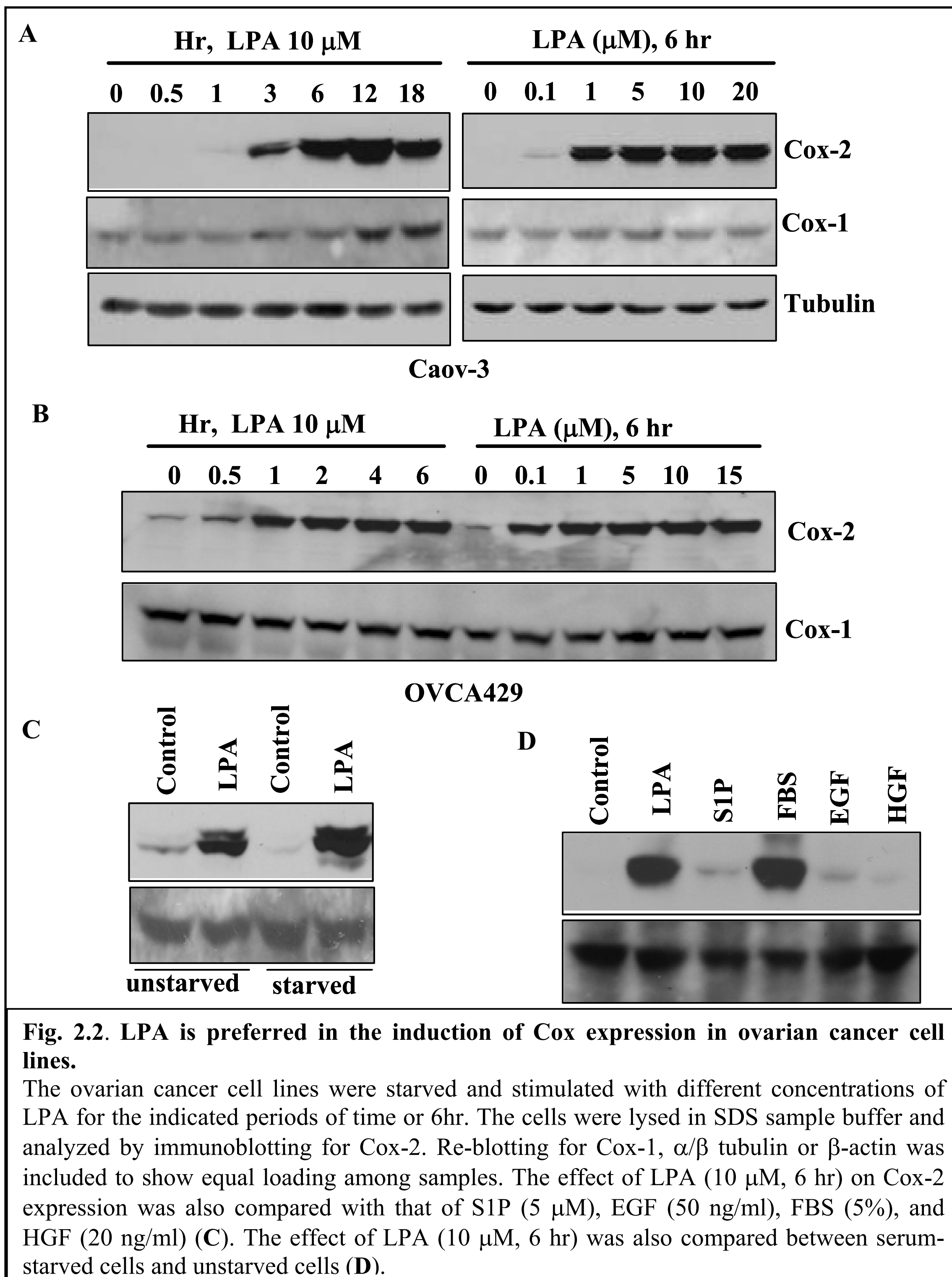


Fig. 2.2. LPA is preferred in the induction of Cox expression in ovarian cancer cell lines.

The ovarian cancer cell lines were starved and stimulated with different concentrations of LPA for the indicated periods of time or 6hr. The cells were lysed in SDS sample buffer and analyzed by immunoblotting for Cox-2. Re-blotting for Cox-1, α/β tubulin or β-actin was included to show equal loading among samples. The effect of LPA (10 μM, 6 hr) on Cox-2 expression was also compared with that of S1P (5 μM), EGF (50 ng/ml), FBS (5%), and HGF (20 ng/ml) (**C**). The effect of LPA (10 μM, 6 hr) was also compared between serum-starved cells and unstarved cells (**D**).

2.3.2 LPA induces PGE_2 production and AA release

We examined whether LPA-induced Cox-2 enzyme is functionally active, contributing to biosynthesis and release of PGE_2. As shown in Fig. 2.3, LPA treatment strongly increased PGE_2 levels in culture supernatants in a dose-dependent manner. LPA treatment led to significant release of AA (Fig. 2.4 A), supplying substrate for production of PGE_2 in LPA-stimulated cells. Further, addition of exogenous AA (10 μM) to unstimulated Caov-3 cells also resulted in significant PGE_2 generation (Fig. 2.4 B), suggesting the cells could utilize the basal Cox enzyme(s) to synthesize PGE_2 when the substrate becomes available.

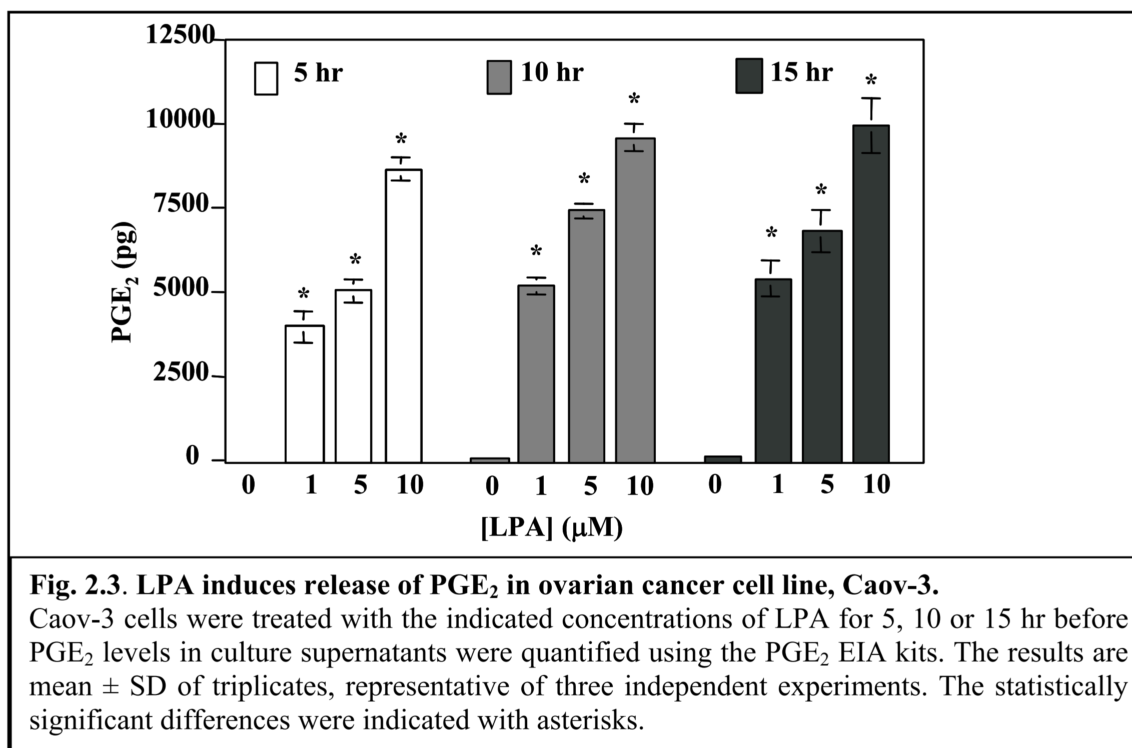


Fig. 2.3. LPA induces release of PGE_2 in ovarian cancer cell line, Caov-3.
Caov-3 cells were treated with the indicated concentrations of LPA for 5, 10 or 15 hr before PGE_2 levels in culture supernatants were quantified using the PGE_2 EIA kits. The results are mean ± SD of triplicates, representative of three independent experiments. The statistically significant differences were indicated with asterisks.

We also observed a synergism between LPA treatment and exogenous AA in stimulation of PGE_2 production (Fig. 2.4 B). However, these effects of LPA, AA and LPA plus AA on PGE_2 generation were all highly sensitive to the Cox-2 enzyme inhibitor NS-398 (Fig. 2.4 C). NS-398 did not affect LPA-induced Cox-2 protein expression (Fig. 2.4 D). These results suggest that LPA activates both AA release and Cox-2 expression. The two processes cooperate to upregulate PGE_2 levels in LPA-treated ovarian cancer cells.

2.3.3 The LPA_1, LPA_2 and LPA_5 receptors mediate LPA-induced Cox-2 expression

To identify the LPA receptors responsible for Cox-2 induction, we assessed expression of LPA receptors in ovarian cancer cell lines by Northern blotting and RT-PCR. Caov-3 and OVCA-429 expressed mRNAs of the LPA_{1-3} receptors and the newly identified LPA_5 receptor (Fig. 2.5). The RT-PCR product of LPA_4 mRNA was not detected in Caov-3 cells and therefore was not further assessed for its role in Cox-2 induction. We utilized small interfering RNA (siRNA) to downregulate expression of each of the expressed LPA receptors in Caov-3 cells (Fig. 2.6 A). The expression of LPA_{1-3} and LPA_5 mRNAs was decreased by 60-80% as determined by RT-qPCR (Fig. 2.6 B). Downregulation of LPA_1, LPA_2 or LPA_5 caused a significant suppression of LPA-induced Cox-2 expression (Fig. 2.6 A). In contrast, downregulation of LPA_3 did not affect LPA-dependent induction of Cox-2 (Fig. 2.6 A).

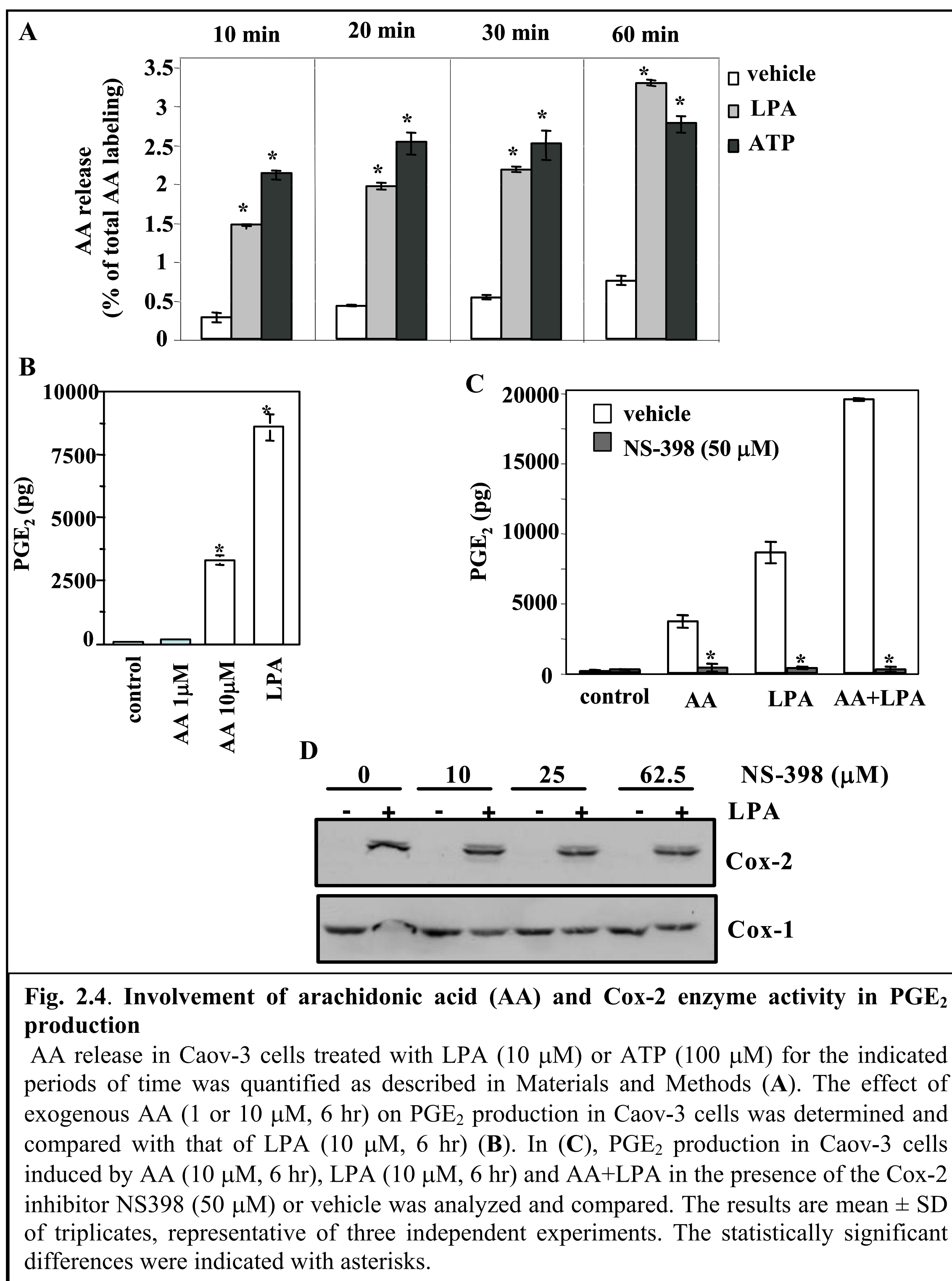


Fig. 2.4. **Involvement of arachidonic acid (AA) and Cox-2 enzyme activity in PGE_2 production**

AA release in Caov-3 cells treated with LPA (10 µM) or ATP (100 µM) for the indicated periods of time was quantified as described in Materials and Methods (**A**). The effect of exogenous AA (1 or 10 µM, 6 hr) on PGE_2 production in Caov-3 cells was determined and compared with that of LPA (10 µM, 6 hr) (**B**). In (**C**), PGE_2 production in Caov-3 cells induced by AA (10 µM, 6 hr), LPA (10 µM, 6 hr) and AA+LPA in the presence of the Cox-2 inhibitor NS398 (50 µM) or vehicle was analyzed and compared. The results are mean ± SD of triplicates, representative of three independent experiments. The statistically significant differences were indicated with asterisks.

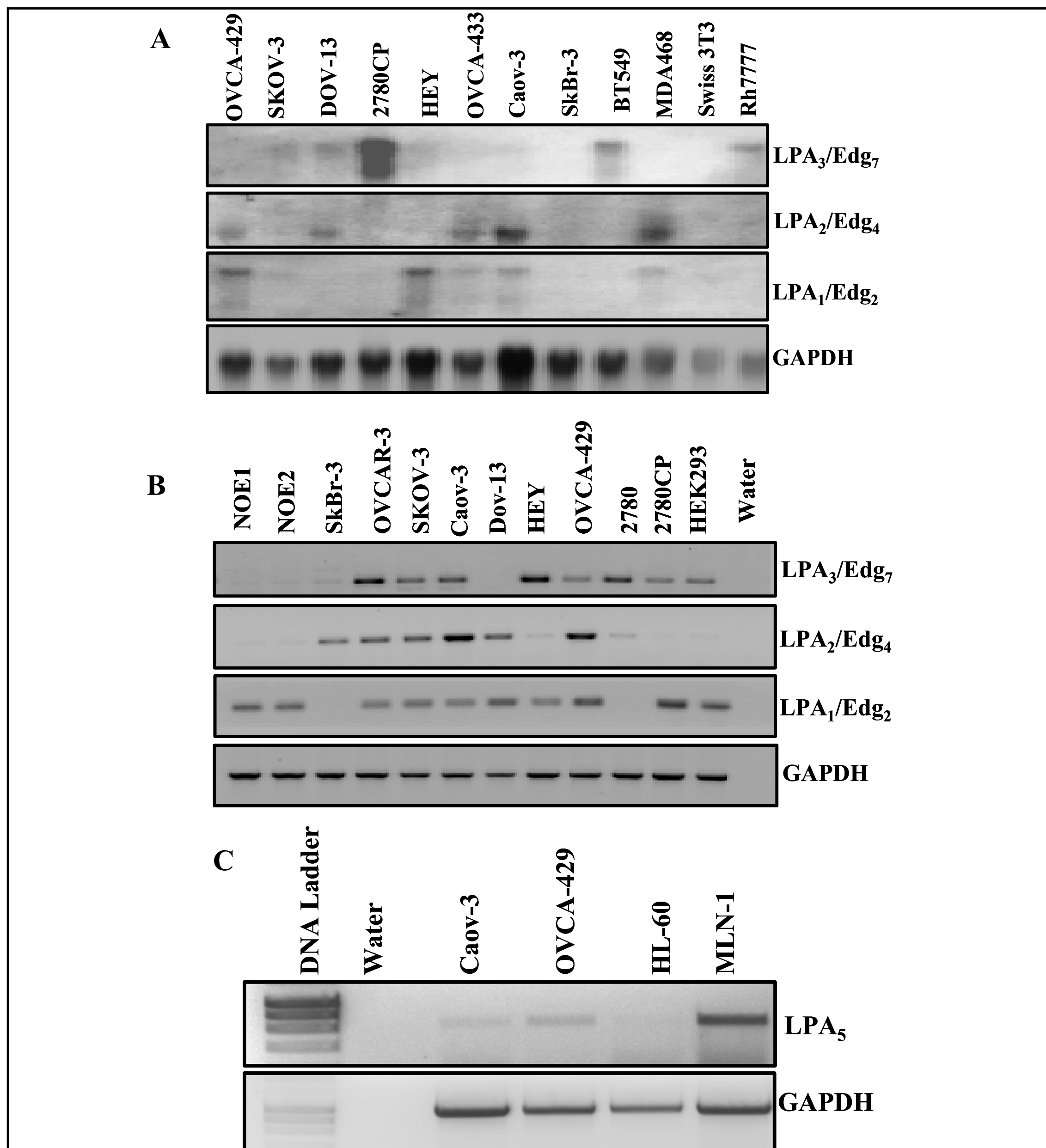


Fig. 2.5. **Expression of LPA receptor subtypes of the Edg family in ovarian cancer cells. A,** Northern blotting analysis of LPA receptor various cell lines was done labeled cDNA probes of LPA_1, LPA_2 and LPA_3. GAPDH was used as loading control. **B**, the relative expression levels of LPA receptors in various normal and epithelial ovarian cancer cells were confirmed by RT-PCR. Other cells of epithelial origin were included for comparison. In **C,** RT-PCR was used to analyze the expression of novel LPA receptor, LPA_5 in ovarian cancer cells. Lung cancer cells (HL-60) and gastric cancer cells (MLN-1) included for comparison.

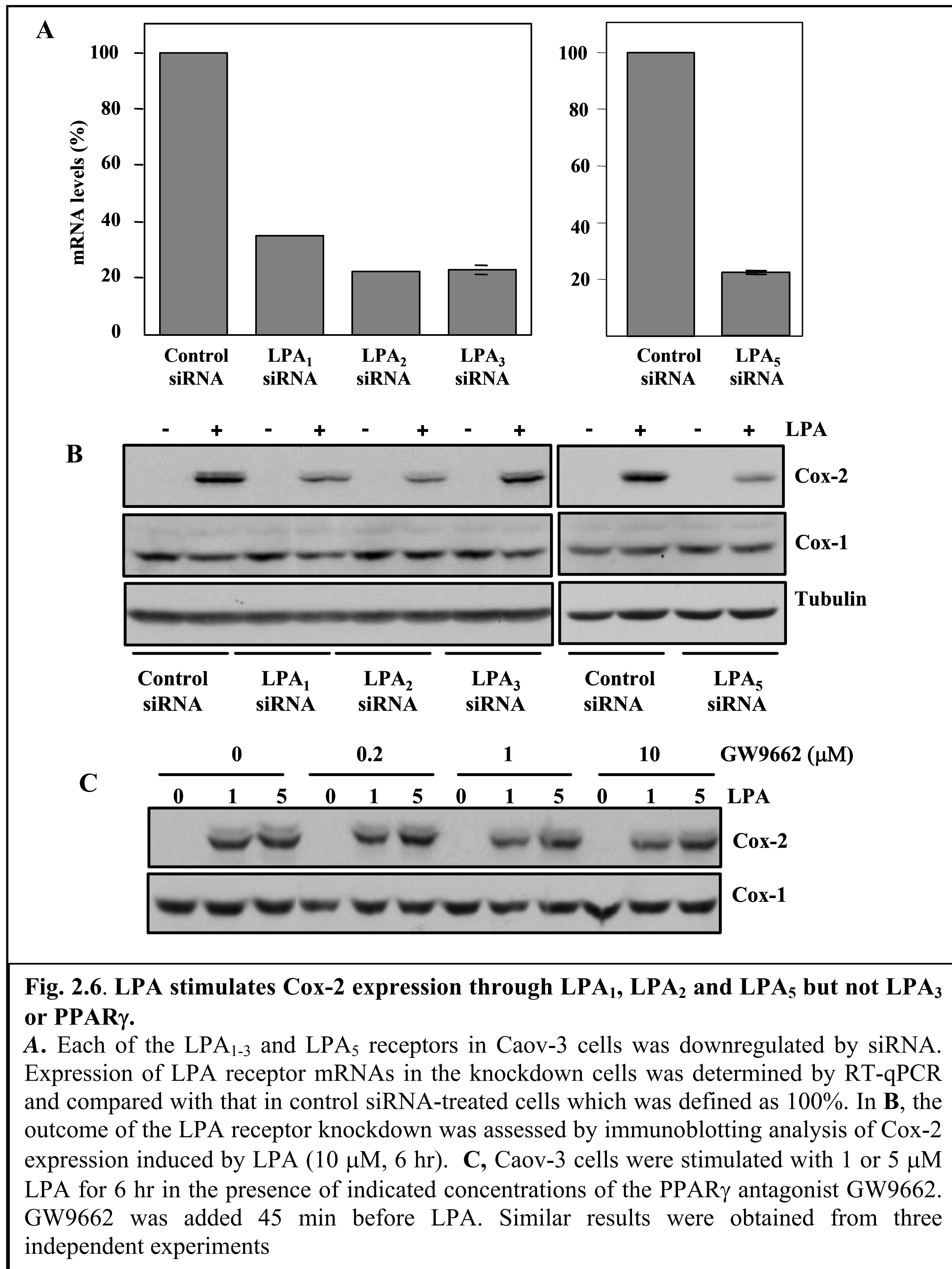


Fig. 2.6. LPA stimulates Cox-2 expression through LPA_1, LPA_2 and LPA_5 but not LPA_3 or PPARγ.
A. Each of the $LPA_{1\text{-}3}$ and LPA_5 receptors in Caov-3 cells was downregulated by siRNA. Expression of LPA receptor mRNAs in the knockdown cells was determined by RT-qPCR and compared with that in control siRNA-treated cells which was defined as 100%. In **B**, the outcome of the LPA receptor knockdown was assessed by immunoblotting analysis of Cox-2 expression induced by LPA (10 μM, 6 hr). **C,** Caov-3 cells were stimulated with 1 or 5 μM LPA for 6 hr in the presence of indicated concentrations of the PPARγ antagonist GW9662. GW9662 was added 45 min before LPA. Similar results were obtained from three independent experiments

PPARγ can stimulate Cox-2 expression through activating the PPARγ binding sites located in the human Cox-2 gene promoter [202]. To address the possibility that LPA may stimulate Cox-2 expression via PPARγ, we treated Caov-3 cells with LPA in the presence of the PPARγ antagonist GW9662 [202]. GW9662 had little effect, if any, on LPA-afforded Cox-2 expression (Fig. 2.6 C), indicating that LPA induces Cox-2 expression through a PPARγ-independent pathway.

2.3.4 LPA induced Cox-2 gene expression does not depend on G_i, ERK or p38

The LPA_1 and LPA_2 receptor subtypes couples to the G_i α subunit of the trimeric G proteins [204-206]. A number of biological actions of LPA are mediated by G_i-dependent signaling cascades including LPA-induced GROα expression as we described recently [192]. To investigate the potential role of G_i in LPA-regulated Cox-2 expression, we stimulated Caov-3 cells that were pre-treated with pertussis toxin (PTX), a specific inhibitor of G_i. LPA was fully capable of inducing Cox-2 expression in cells treated with PTX, indicating that LPA-induced Cox-2 expression is independent of G_i signaling cascades (Fig. 2.7). We also excluded the involvement of ERK or p38MAPK in the process, by using the pharmacological inhibitor of MEK (PD98059) and p38MAPK (SB203580) (data not shown). LPA stimulated a full magnitude of Cox-2 expression in the presence of various concentrations of these inhibitors (Fig. 2.7). These results suggest a novel pathway independent of Gi and MAPKs in LPA activation of the Cox-2 gene expression.

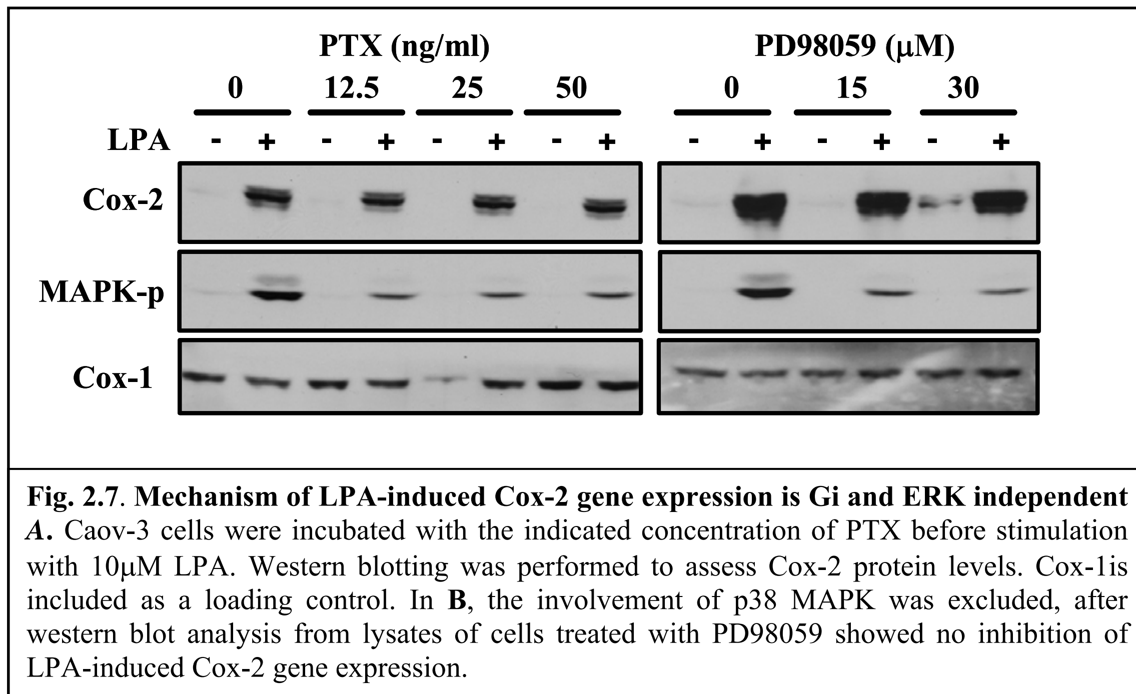


Fig. 2.7. **Mechanism of LPA-induced Cox-2 gene expression is Gi and ERK independent** ***A.*** Caov-3 cells were incubated with the indicated concentration of PTX before stimulation with 10μM LPA. Western blotting was performed to assess Cox-2 protein levels. Cox-1is included as a loading control. In **B**, the involvement of p38 MAPK was excluded, after western blot analysis from lysates of cells treated with PD98059 showed no inhibition of LPA-induced Cox-2 gene expression.

2.3.5 The effect of LPA involves both transcriptional activation and post-transcriptional enhancement of Cox-2 mRNA stability

Cox-2 is one of the immediate early response genes and its induction usually declines rapidly within 1-4 hours [207]. However, LPA-mediated Cox-2 protein expression lasted much longer in ovarian cancer cells and was detectable at high levels 12-18 hours after addition of LPA (Fig. 2.2). Consistent with this, Cox-2 mRNA levels reached the maximum at 6 hours and remained highly elevated at 12 hours (Fig. 2.8 A). The sustained induction of Cox-2 mRNA by LPA suggests that Cox-2 mRNA could be stabilized in LPA-treated cells. To examine this possibility, we compared the decay kinetics of Cox-2

transcripts in Caov-3 cells treated with or without LPA. As shown in Fig. 2.8 B, Cox-2 transcripts in control cells degraded quickly after actinomycin D was added to halt new RNA synthesis. In contrast, pretreatment with LPA led to significant stabilization of Cox-2 mRNA as compared to untreated control cells (Fig. 2.8 B).

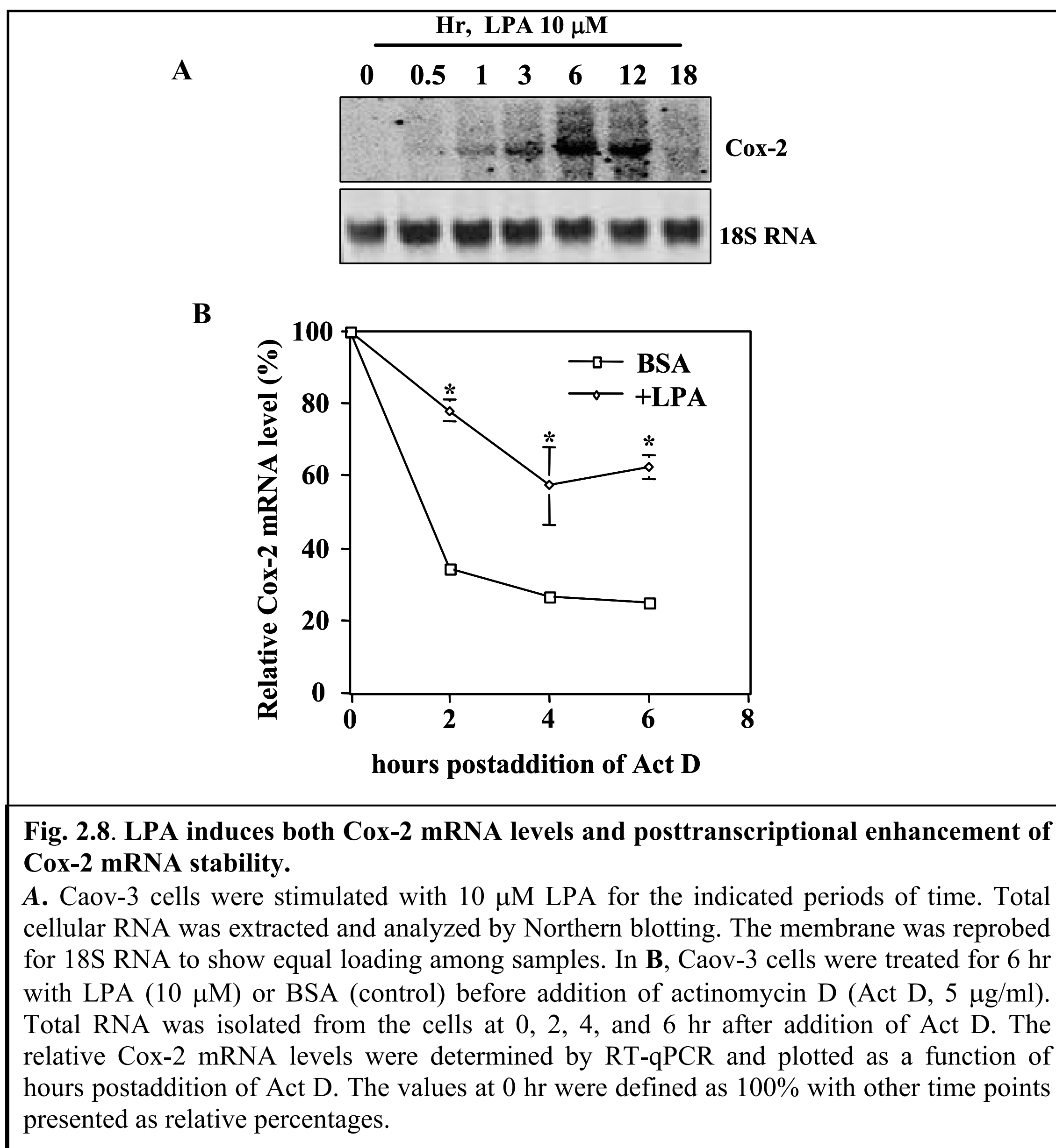


Fig. 2.8. **LPA induces both Cox-2 mRNA levels and posttranscriptional enhancement of Cox-2 mRNA stability.**
A. Caov-3 cells were stimulated with 10 μM LPA for the indicated periods of time. Total cellular RNA was extracted and analyzed by Northern blotting. The membrane was reprobed for 18S RNA to show equal loading among samples. In **B**, Caov-3 cells were treated for 6 hr with LPA (10 μM) or BSA (control) before addition of actinomycin D (Act D, 5 μg/ml). Total RNA was isolated from the cells at 0, 2, 4, and 6 hr after addition of Act D. The relative Cox-2 mRNA levels were determined by RT-qPCR and plotted as a function of hours postaddition of Act D. The values at 0 hr were defined as 100% with other time points presented as relative percentages.

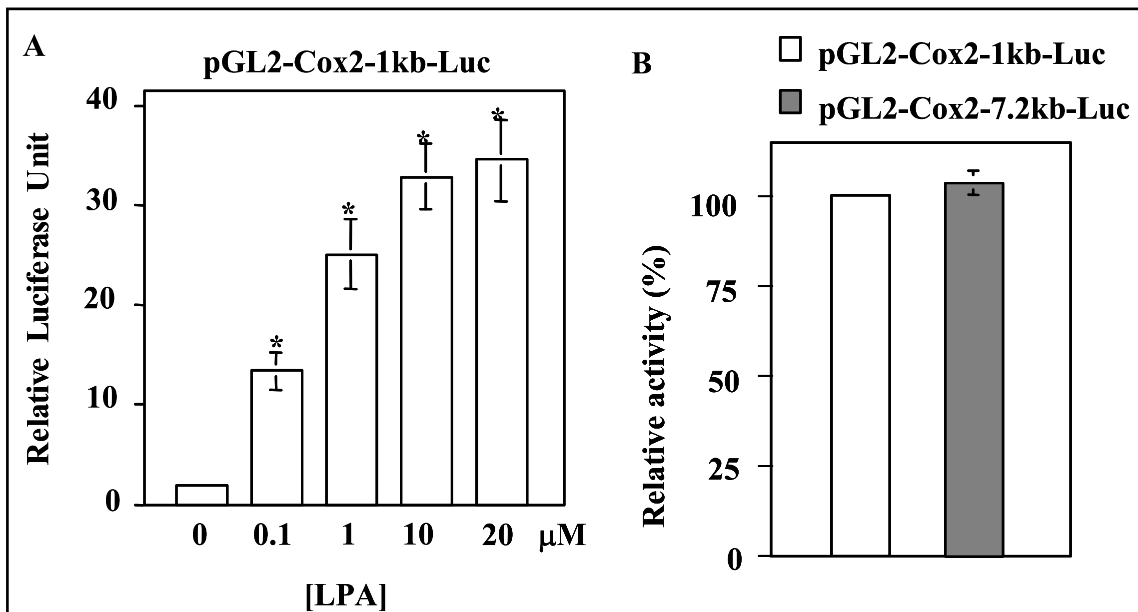


Fig. 2.9. Effect of LPA on Cox-2 expression involves transcriptional activation of Cox-2 promoter
Caov-3 cells transfected with pGL2-Cox2-1kb-Luc containing the –980-+15 fragment of the Cox-2 promoter were stimulated with LPA at the indicated concentrations for 6 hours and assayed for luciferase activity (**A**) LPA-induced luciferase activity from Caov-3 cells transfected with pGL2-Cox2-1kb-Luc (defined as 100%) was compared with the activity from the cells transfected with pGL2-Cox2-7.2bk-Luc (**B**). All numeric results were mean ± SD of triplicates, representative of three independent experiments. The statistically significant differences of the data were indicated with asterisks.

To determine whether LPA induction of Cox-2 mRNA was initiated from transcriptional activation, we cloned an approximately 1 kb proximal fragment (-980 to +15) of the human Cox-2 gene promoter and evaluated its ability to drive transcription of the luciferase reporter in response to LPA. LPA stimulated 5-20 fold increases in luciferase activity in Caov-3 (Fig. 2.9 A). In addition, we compared the response of this reporter with that of a luciferase vector containing 7.2 kb 5' flanking region (pGL2-Cox2-7.2kb-Luc) [202]. Similar ranges of LPA-stimulated luciferase activity were obtained from each

of the two plasmids, suggesting that essential LPA-regulatory elements are located within the 1 kb sequences of the Cox-2 promoter (Fig. 2.9 B).

2.3.6 LPA induces transcriptional activation of Cox-2 via C/EBP

To identify the transcription factors driving Cox-2 expression, we analyzed the Cox-2 gene promoter by deletion and mutation of the regulatory cis elements. Within the 1 kb region that responded well to LPA, there are numerous transcription factor binding sites, including those for AP-1, NF-κB, and C/EBP (Fig. 2.10). Deletion of the unique AP-1-like site at around -577 did not attenuate the response to LPA as determined by luciferase assays (Fig. 2.10). In agreement with this, ectopic expression of TAM67, a dominant negative form of c-Jun [208], did not inhibit LPA-induced Cox-2 (Fig. 2.11 A).

Similarly, mutation of the two NF-κB consensus sites did not interfere with the responsiveness of the promoter to LPA (Fig. 2.10 A). Recent studies suggest that LPA and other GPCR agonists stimulate NF-κB activation through the CARMA3/Bcl10/Malt1 signalosome, a process similar to antigen receptor-mediated NF-κB activation in lymphocytes [209, 210].

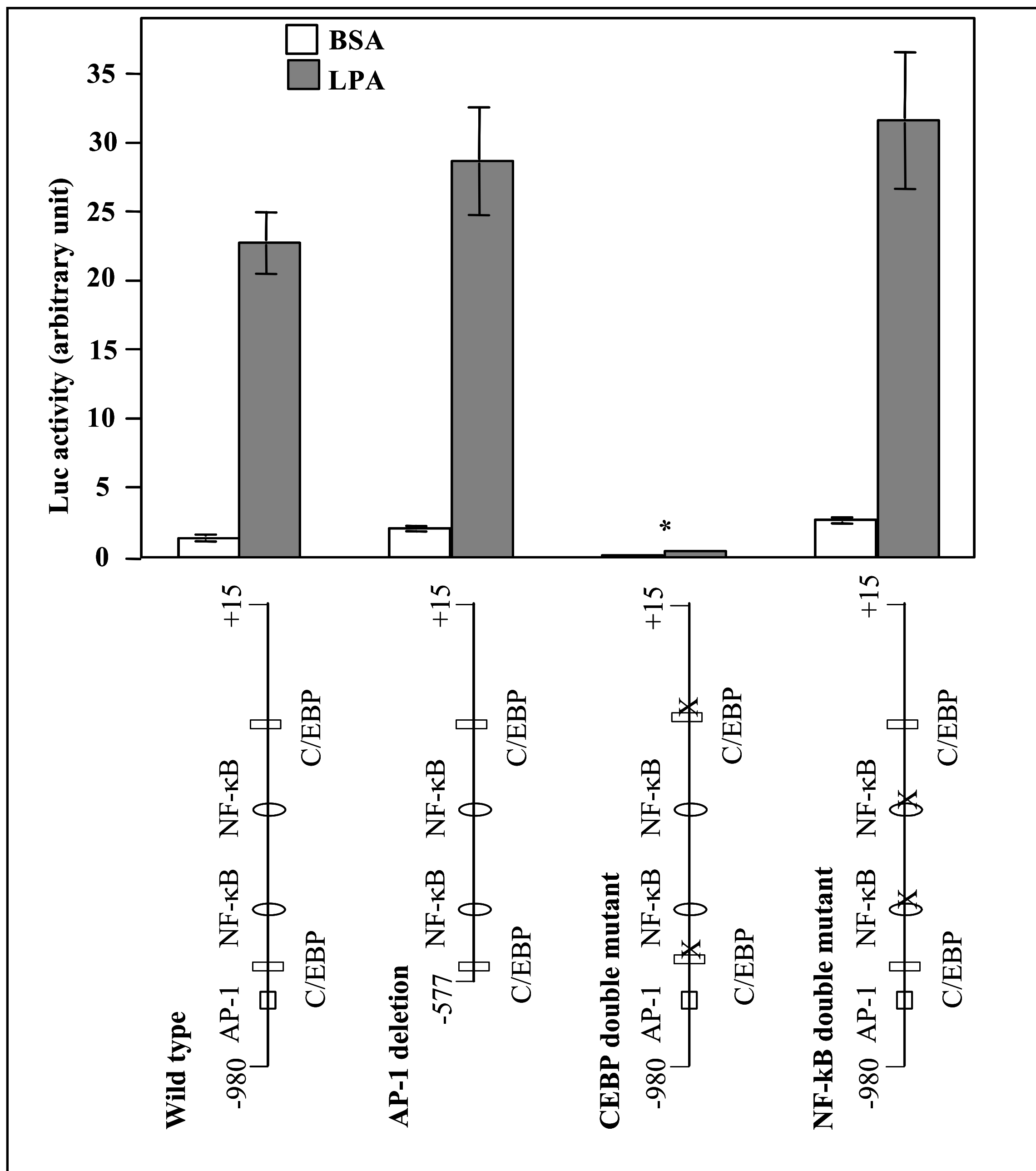
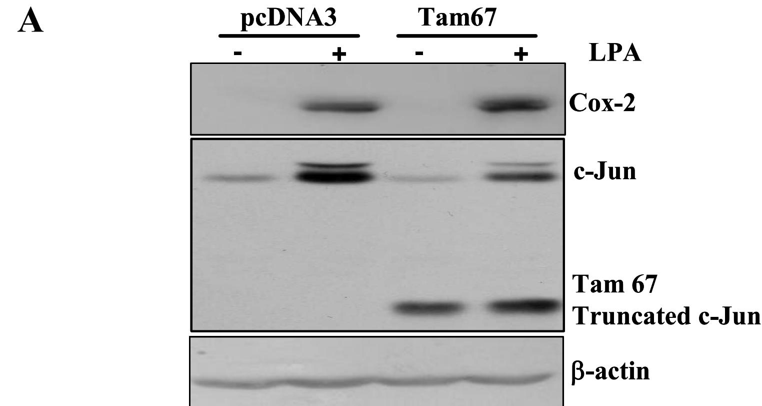


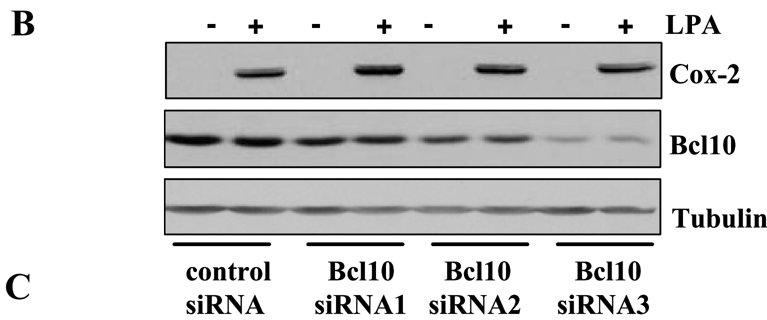
Fig. 2.10. LPA induces transcriptional activation of Cox-2 through C/EBP independently of AP-1 or NF-κB.

The unique AP-1-like, two NF-kB and two C/EBP binding sites were deleted or point mutated as detailed in Materials and Methods. Caov-3 cells transfected with the wild type or mutant constructs were treated with LPA for 6 hours and assayed for luciferase activity. tool.Data shown are representative of three independent experiments.

To confirm the mutagenesis results, we downregulated the expression of Bcl10 with three individual siRNAs. Each significantly reduced Bcl10 expression. However, none of these siRNAs was able to prevent LPA-mediated Cox-2 expression (Fig. 2.11 B). In contrast, the Bcl10 siRNAs markedly inhibited LPA-stimulated IL-8 production (Fig. 2.11 C), a response fully dependent on NF-κB activation as we described previously [9]. The results suggest that in sharp contrast to many other Cox-2 inducers, LPA stimulated Cox-2 expression independently of NF-κB although LPA potently induced NF-κB DNA-binding and the transcriptional activity in these cells (Fig. 4.5 of Chapter 4).

We next targeted the two C/EBP consensus motifs present in the 1 kb fragment of the Cox-2 promoter. Although mutation of the individual site only slightly decreased the promoter activity, simultaneous mutation of the two C/EBP sites resulted in almost complete loss of LPA-induced luciferarse activity (Fig. 2.10), suggesting that these C/EBP-binding sites are essential for the transcriptional activation of Cox-2 by LPA. In further support of this, co-expression of LIP, a dominant negative, truncated form of C/EBP-β [139], inhibited LPA-induced luciferase activity by 60% in cells transfected with pGL-2-Cox2-1kb-Luc (Fig. 2.12 A). LIP also suppressed LPA-induced Cox-2 protein expression when transiently transfected and expressed in Caov-3 cells (Fig. 2.12 B). ChIP assay confirmed the recruitment of C/EBP-β to the Cox-2 gene promoter following LPA treatment (Fig. 2.12 C). Treated or untreated Caov-3 cell lysates were subjected to immunoprecipitation with anti-C/EBP-β and gel eletrophoretic analysis of precipitated DNA using Cox-2 promoter specific primers. As shown in Fig. 2.12 C, LPA induced a rapid localization of C/EBP-β to Cox-2 promoter sequences.





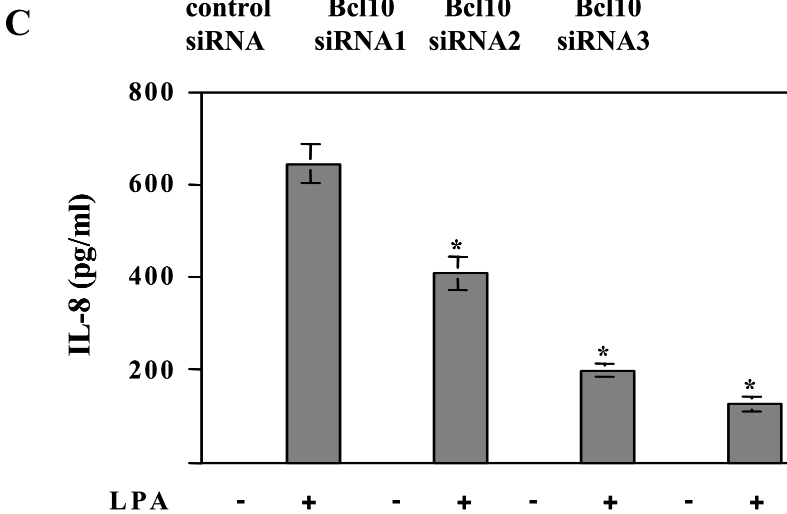


Fig. 2.11. **Inhibition of AP-1 or NF-κB does not interfere with LPA-induced Cox-2 gene expression.**

In A, Caov-3 cells were transfected with pcDNA3-Tam67 or pcDNA3 using Amaxa nucleofector, stimulated for 6 hr with LPA (10 μM) and analyzed by immunoblotting for Cox-2. Control and Bcl10 siRNA-treated Caov-3 cells were stimulated for 6 hr with 10 mM LPA or vehicle. Cox-2 expression in cell lysates (**B**) and IL-8 concentrations (**C**) in culture supernatants were determined by immunoblotting and ELISA analysis, respectively.

C/EBP-β, a major isoform of the C/EBP family, was previously reported to be overexpressed in ovarian cancers [135]. To assess the effect of overexpression of C/EBP-β on activity of the Cox-2 promoter, we transfected Caov-3 cells with pGL2-Cox2-1kb-Luc along with the C/EBP-β LAP1, LAP2 or a control vector. Expression of the C/EBP-β LAP1 or LAP2 was not sufficient to induce activation of luciferase activity from pGL2-Cox2-1kb-Luc (data not shown). The observation suggests that LPA induced Cox-2 expression requires C/EBP activation rather than changes in C/EBP-β protein levels.

2.3.7 The mRNA-binding protein HuR associates with and stabilizes Cox-2 mRNA in LPA-treated cells

There are several regulatory mechanisms to control mRNA stability under different physiological and pathophysiological conditions [211, 212]. One of such regulations involves the RNA-binding protein HuR that associates with AUUUA repeats present in the 3' UTR of mRNAs encoding cytokines and angiogenic factors [212, 213]. Two major Cox-2 transcripts (2.8 kb and 4.6 kb) are derived from alternative polyadenylation of the Cox-2 gene [212, 214].

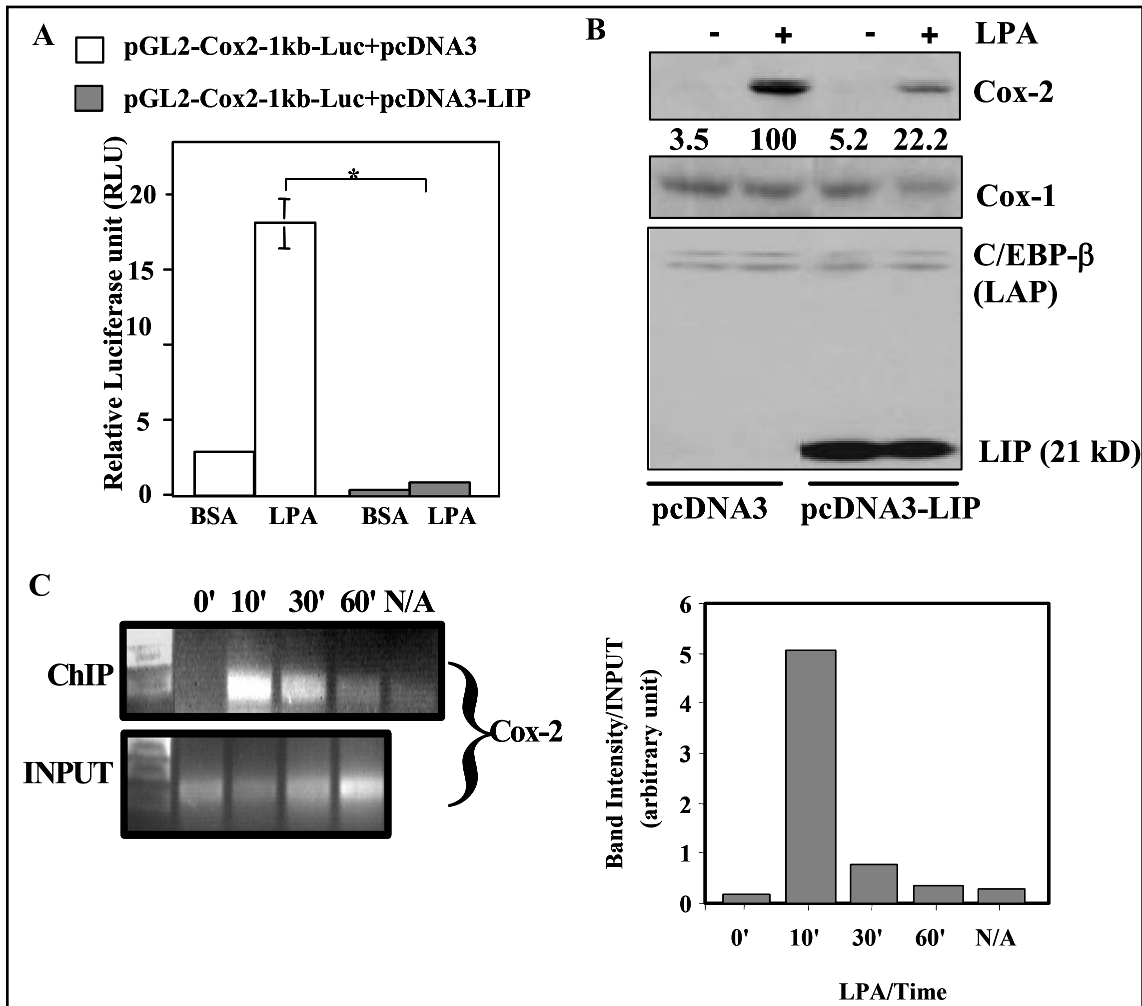


Fig. 2.12. **C/EBP-β is crucial for transcriptional activation of Cox-2 expression by LPA** Caov-3 cells transfected with pGL2-Cox2-1kb-Luc along with pcDNA3 or pCDNA3-LIP were treated for 6 hr with LPA (10 μM) and assayed for luciferase activity (**A**)**.** In **B**, Caov-3 cells were transfected with pcDNA3-LIP or pcDNA3 using Amaxa nucleofector, stimulated for 6 hr with LPA (10 μM) and analyzed by immunoblotting for Cox-2 and C/EBP-β. The values beneath each lane represent relative intensities (%) quantified by densitometry with Cox-2 induced by LPA in pcDNA3-transfected cells defined as 100% with other time pointspresented as relative percentages. (**C**) The presence of C/EBP-β near Cox-2 promoter was confirmed by ChIP assay (see Materials and Methods), by immunoprecipitation of control or 10μM LPA-treated Caov-3 cell lysate with anti-C/EBP-β or mouse IgG (N/A). Image of gel electrophoresis analysis of precipitated Cox-2 DNA fragment was quantified by NIH Image J

In ovarian cancer cells, LPA induced the 4.6 kb transcript which contains a long 3'-UTR [212, 214]. The 2.8 kb transcript lacking the 3'-UTR was not induced at detectable levels by LPA (Fig. 2.8 A). Importantly, multiple AUUUA repeats are present within the 3'-UTR of the 4.6 kb Cox-2 transcript [212, 215]. Moreover, HuR is highly expressed in primary ovarian cancers and ovarian cancer cell lines [216]. These observations prompted us to examine whether HuR participates in protection of Cox-2 mRNA stability, contributing to the persistent induction of Cox-2 observed in LPA-stimulated cells.

We first examined whether HuR physically binds to Cox-2 mRNA *in vitro*. To this end, the 3'UTR of Cox-2 mRNA was synthesized and labeled with biotin by *in vitro* transcription. As demonstrated in Fig. 2.13 A, the Cox-2 3'-UTR was capable of binding with HuR protein from lysates of Caov-3 cells. Of note, incubation of 90 μg cellular protein with 1.5 μg Cox-2 3'-UTR brought down amounts of HuR similar to that present in 30 μg cell lysates (Fig. 2.13 A). Therefore, approximately one third of the total HuR protein formed complex with the exogenous Cox-2 3'-UTR, demonstrating that HuR has a strong binding affinity for the Cox-2 3'-UTR at least in *in vitro* binding assays.

To confirm that the HuR-Cox-2 mRNA association occurs within the cell, we immunoprecipitated HuR from Caov-3 cells treated with LPA or BSA for 6 hours. The immunocomplexes were then subjected to reverse transcription followed by PCR amplification of a 481bp fragment of Cox-2 cDNA. As shown in Fig. 2.13B, the fragment was detected in immunoprecipitates of the cells treated with LPA, but not present in that of control cells. It was also absent from immunoprecipitates of LPA-treated cells when a control antibody was used to replace the specific HuR antibody. HuR therefore indeed

formed complex with Cox-2 mRNA in LPA-treated cells. The lack of Cox-2 mRNA in the immunoprecipitates of control cells suggests that the interaction between HuR and Cox-2 mRNA may not be constitutive. It could be regulated through activation of HuR protein or rely on LPA-dependent increases in Cox-2 mRNA abundance.

To determine the functional significance of HuR binding to Cox-2 mRNA, we assessed the effect of downregulation of HuR on LPA-induced Cox-2 expression. Knockdown of HuR expression resulted in a significant decrease in LPA-induced Cox-2 as shown in Fig. 2.13 C. Stronger suppression of Cox-2 expression was observed in cells treated with LPA for 12 hours compared to the cells treated with LPA for 6 hours. The observation suggests that HuR-mediated stabilization of Cox-2 mRNA contributes more significantly to Cox-2 expression when transcriptional activation becomes less active after prolonged incubation with LPA.

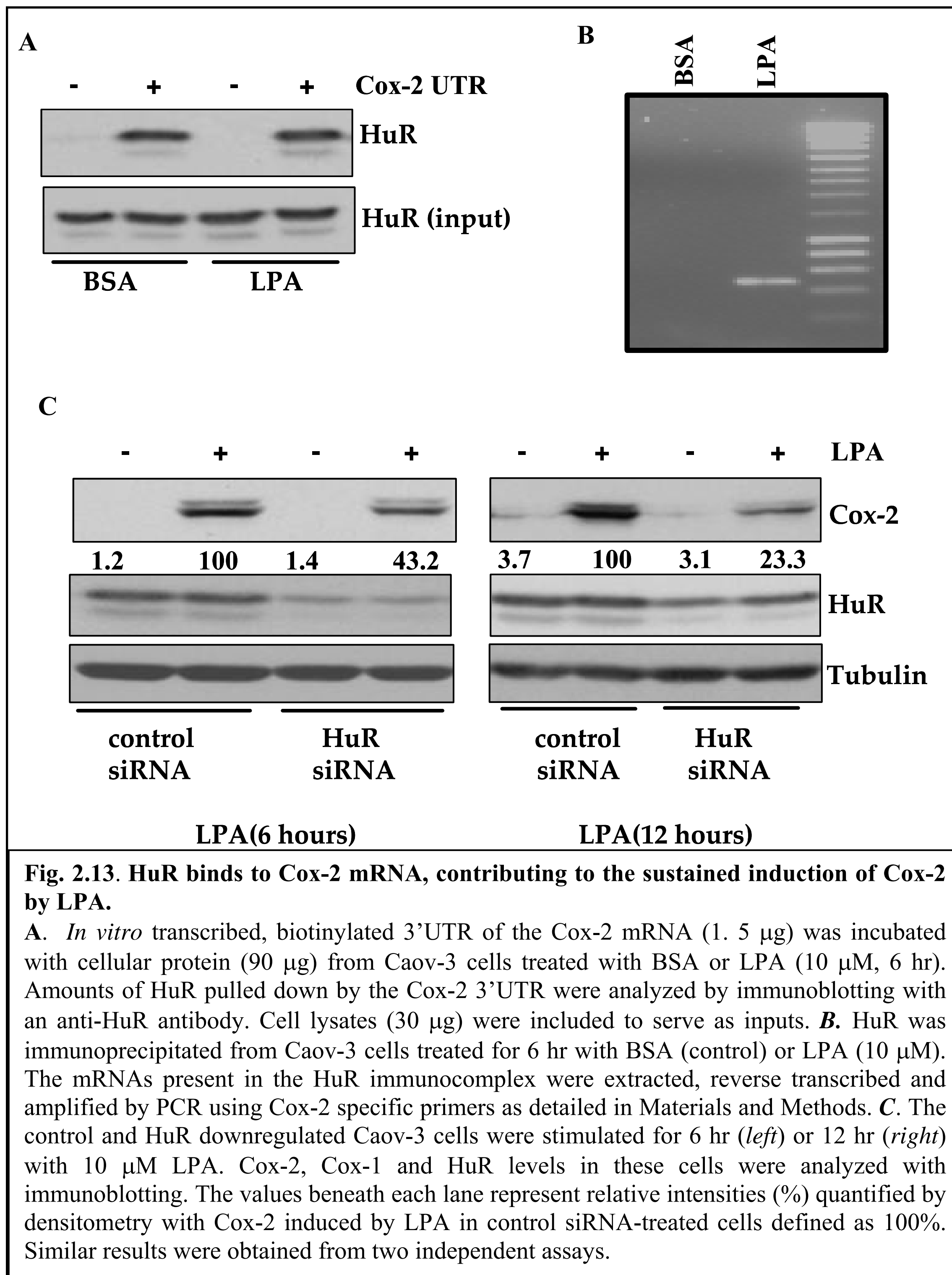


Fig. 2.13. **HuR binds to Cox-2 mRNA, contributing to the sustained induction of Cox-2 by LPA.**

A. *In vitro* transcribed, biotinylated 3'UTR of the Cox-2 mRNA (1. 5 µg) was incubated with cellular protein (90 µg) from Caov-3 cells treated with BSA or LPA (10 µM, 6 hr). Amounts of HuR pulled down by the Cox-2 3'UTR were analyzed by immunoblotting with an anti-HuR antibody. Cell lysates (30 µg) were included to serve as inputs. ***B.*** HuR was immunoprecipitated from Caov-3 cells treated for 6 hr with BSA (control) or LPA (10 µM). The mRNAs present in the HuR immunocomplex were extracted, reverse transcribed and amplified by PCR using Cox-2 specific primers as detailed in Materials and Methods. ***C***. The control and HuR downregulated Caov-3 cells were stimulated for 6 hr (*left*) or 12 hr (*right*) with 10 µM LPA. Cox-2, Cox-1 and HuR levels in these cells were analyzed with immunoblotting. The values beneath each lane represent relative intensities (%) quantified by densitometry with Cox-2 induced by LPA in control siRNA-treated cells defined as 100%. Similar results were obtained from two independent assays.

2.4 Discussion

In the present study, we demonstrated that LPA induces robust and sustained expression of Cox-2 in ovarian cancer cells through both transcriptional and post-transcriptional mechanisms, mediated by the C/EBP transcription factor and the mRNA stability protein HuR, respectively. The identification of C/EBP as a primary transcription factor responsible for LPA-induced Cox-2 expression is somewhat surprising, as C/EBP has not been previously linked to any biological actions of LPA. Other transcription factors activated by LPA and commonly involved in inducible Cox-2 expression such as NF-κB and AP-1 are instead dispensable for LPA-induced Cox-2. Previous studies of GPCR regulation of Cox-2 in different cell systems have led to diverse mechanisms involving multiple transcription factors [217, 218]. A predominant and specific role of C/EBP in transcriptional activation of Cox-2 via GPCR signaling has not been previously described and likely represents a general mechanism regulating Cox-2 expression by LPA and other GPCR agonists in different cell types.

Since C/EBP-β expression is elevated in primary ovarian cancers and in ovarian cancer cell lines [135], the isotype likely plays a major role in mediating transcriptional activation of Cox-2 in response to LPA. C/EBP-β has been shown to be a key transcription factor in regulation of Cox-2 expression by aspirin and salicylate [219]. Furthermore, expression of other isotypes of C/EBP such as C/EBP-α and C/EBP-δ is limited or undetectable in ovarian cancer cell lines (data not shown). We did not observe activation of the Cox-2 promoter by means of transfection of exogenous C/EBP-β, suggesting that

overexpression of C/EBP-β protein is insufficient to confer the transcriptional activation of Cox-2. The luciferase reporter analysis established that LPA treatment stimulates C/EBP transcriptional activity. There are multiple post-translational modifications associated with C/EBP activation including phosphorylation [220], acetylation [221] and sumoylation [201, 222]. Treatment with LPA resulted in prominent phosphorylation of C/EBP-β. It is not known whether LPA-dependent activation of C/EBP is also regulated by acetylation and sumoylation in addition to phosphorylation.

LPA-induced Cox-2 gene expression is mediated by LPA_1, LPA_2 and LPA_5 receptors independent of LPA_3 and LPA_4. The LPA_1 receptor is the most commonly expressed subtype present in both normal and cancerous tissues [62]. The LPA_2 receptor subtype is abnormally overexpressed in ovarian cancers and other human malignancies [61, 62, 64]. It mediates LPA-dependent cytokine production in ovarian and breast cancer cells [9, 192]. Similarly, Hu et al. described that LPA_2 expression correlates with the ability of LPA to induce VEGF expression in ovarian cancer cells [193]. The importance of LPA_2 in modulation of gene expression is further highlighted by the observation that transgenic expression of LPA_2 driven by an ovary-selective promoter led to the production of higher levels of VEGF and uPA mRNA and proteins in ovaries of transgenic mice [223]. In addition to the role in modulation of gene expression, LPA_2 may also regulate cell motility [224, 225]. Involvement of LPA_5 in LPA-induced Cox-2 expression is an interesting observation since the biological functions of this new LPA receptor are totally unknown [40]. Requirement of LPA_1, LPA_2 and LPA_5 in LPA induction of Cox-2 suggests that these LPA receptors could each contribute to the response to LPA. Alternatively, the effect of

LPA may depend on combined functions of these receptors. A recent study suggests that these LPA receptors can crosstalk to each other through forming heterodimers with signaling properties likely different from their homodimers [226]. In addition, the LPA_3 receptor does not seem to be a mediator of Cox-2 induction in ovarian cancer cells although this receptor is highly expressed in most ovarian cancer cell lines [62]. The result is inconsistent with the critical role of LPA_3 in the uterine Cox-2 expression as suggested by studies of LPA_3 knockout mice [199], indicating different LPA receptor subtypes are capable of mediating Cox-2 expression depending on the cellular context.

The proximal region of the 3'-UTR of COX-2 mRNA contains highly conserved AU-rich elements that have potential to interact with multiple mRNA binding proteins including β-catenin, TIAR, AUF1, HuR, hnTIA-1 and hnRNP [211, 212, 215]. In the current study, we focused on the role of HuR, a member of the ELAV (embryonic lethal abnormal vision) family of mRNA-binding proteins [213]. HuR overexpression is associated with increased levels of Cox-2 protein in cancers of the colon, stomach, breast and ovary [135, 212, 227, 228]. However, few studies have provided direct evidence that HuR plays a causal role in Cox-2 overexpression in malignant cells. Our results indicate that HuR physically binds to the Cox-2 3’-UTR and protects Cox-2 mRNA stability. This protein-mRNA association contributes significantly to the sustained induction of Cox-2 by LPA. HuR thus provides a positive feedback to Cox-2 induction during physiological responses to LPA and probably other environmental stimuli.

CHAPTER 3

MECHANISMS FOR ACTIVATION OF C/EBP-β BY LPA

Part of the work presented in this chapter has been published FASEB J. 22:2639-2651 (2008).

3.0 Abstract

We previously showed that LPA induced the expression of Cox-2, one of the enzymes that convert arachidonic acid to prostaglandins. Through site-directed mutagenesis and the ectopic expression of a dominant negative form C/EBP-β, we identified C/EBP-β as an essential transcription factor in the induction of Cox-2 gene expression by LPA. Here, we further examined the precise mechanisms underlying LPA-induced activation of C/EBP-β. LPA induced a rapid and transient phosphorylation of C/EBP-β in ovarian cancer cells. Prolonged exposure to LPA stimulation caused an increase in C/EBP-β protein levels. Using electromobility shift assay, we show a sustained increase in DNA binding activity of C/EBP-β following LPA stimulation. Functionally, LPA induced multifold increase in C/EBP-β transcriptional activity in ovarian cancer cells transfected with a reporter construct of consensus C/EBP binding site. Furthermore, we report a novel mechanism integrating GPCR signals and a permissive activity from a receptor tyrosine kinase (RTK) in LPA-induced phosphorylation and activation of C/EBP-

β. This role of RTK was not consistent with LPA activation of C/EBP-β through transactivation of RTK, as full activation of RTKs by their own agonists only weakly stimulated C/EBP-β activities. Interestingly, the Cox-2 promoter activity and gene expression were also dependent on RTK signaling, further substantiating the regulatory role of C/EBP-β in LPA-induced Cox-2 gene expression. These results together provide a picture of signaling cascades involved in LPA activation of C/EBP-β.

3.1 Introduction

C/EBP-β belongs to a widely expressed, highly conserved family of basic region-leucine zipper (bZIP) class of transcription factors that play essential role in many physiological and pathological processes such as cellular differentiation and inflammation [121, 126, 127]. Other members are C/EBP-α, C/EBP-γ, C/EBP-δ, C/EBP-ε, and C/EBP-ζ. The genes of most C/EBPs are intronless and exhibit a high degree of homology in the basic and leucine zipper regions [120, 121]. However, each C/EBP isoform may exhibit distinct functions. For instance, ablation of the C/EBP-α gene in mice led to neonatal lethality due to hypoglycemia and absence of stored liver glycogen [124, 129]. C/EBP-β-null mice are, however, viable in spite of serious defects in hematopoiesis and the immune system [130, 131]. The C/EBP-β gene is expressed as three isoforms as a consequence of alternative initiation start sites within the C/EBP-β transcript: liver-enriched activating proteins (LAP1—49kd and LAP2—45 kda) and liver-enriched inhibiting protein (LIP—20 kda) [138, 139, 229]. The LAP isoforms function as activators of transcription while LIP,

which lacks the transactivation domain, is a dominant negative transcriptional repressor. The differential expression of these LAP and LIP is regulated by C/EBP-α [140] and has been found to be critical in cell fate determination [141].

Several studies have shown that C/EBP-β is important in mammalian reproduction including the development and differentiation of the mammary glands. C/EBP-β is also involved in ovarian follicular development and corpus leteum formation by enhancing the effects of lutenizing hormone (LH/hCG) [132-134]. Moreover, C/EBP-β null mice showed defective female reproduction [132]. C/EBP-β has been implicated in many cancer types and its expression highly correlates with aggressive behavior in ovarian cancer cells [135]. C/EBP binding sites are present in the promoters of cancer or metastasis-related genes such as interleukin 6 (IL-6), cyclooxygenase 2 (cox-2), urokinase plasminogen activator (uPA) and tumor necrosis factor alpha (TNF-α). In fact, C/EBP-β was identified in pioneer studies as a protein that binds the IL-1 response element on IL-6 promoter and hence, was originally named NF-IL6 [138, 230, 231]. We recently described C/EBP-β as an essential transcription factor for induction of the Cox-2 gene expression by LPA (Chapter 2 , [232]). The exact mechanism by which LPA induces activation of C/EBP-β has not been elucidated.

Recent studies showed that many biological functions of GPCRs depend on EGFR. It is currently believed that ligands for GPCRs such as LPA activate cellular responses through transactivation of EGFR or other highly expressed RTK through the proteolytic release of EGFR ligands such as EGF or HB-EGF. The transactivation model is not

consistent with LPA stimulation of EGFR phosphorylation and activation. In addition, it is not known whether the crosstalk between GPCRs and EGFR involves activation of transcription factors such as C/EBP-β.

Here, we provide evidence that LPA modulates the transcriptional activities of C/EBP-β in ovarian cancer cells by inducing its phosphorylation and DNA binding. LPA also induced expression of C/EBP-β at later time points which may contribute to the sustained induction of Cox-2. The effects of LPA on C/EBP-β phosphorylation, DNA-binding and transcriptional activation required permissive signals from EGFR or an alternate RTK. The dependence of LPA actions on RTK also applies to other C/EBP-β target genes including IL-6 and uPA in addition to Cox-2 underscoring a general role of RTK in GPCR activation of C/EBP-β and C/EBP-β-target genes.

3.2 Materials and Methods

Materials 1-Oleoly (18:1) LPA and sphingosine 1 phosphate (S1P) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Prior to use, these phospholipids were dissolved in PBS containing 0.5% fatty acid-free bovine serum albumin (BSA). BSA, Fugene 6 and protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). [Plasmid DNA was purified using the endo-free purification kit from Qiagen (Valencia, CA). Luciferase assay reagents were obtained from Promega (Madison, WI). All oligonucleotides and primers were synthesized by Operon Biotechnologies, Inc

(Huntsville, AL). QuikChange XL site directed mutageneis kit were purchased from Stratagene (La Jolla, CA). Anti-phospho CEBPβ and anti-tubulin α/β antibodies were obtained from Cell Signaling (Danvers, MA). Insulin and cell culture medium were obtained from Invitrogen Inc. (Carlsbad, CA). Bovine fetal serum was from Biomeda (Foster City, CA). Insulin-like growth factor I (IGF-I) was from Upstate Biotechnology (Lake Placid, NY). Hepatocyte growth factor (HGF) and Quantikine IL-6 ELISA kit were from R & D systems (Minneapolis, MN). Anti-uPA monoclonal antibody #394 was obtained from American Diagnostica (Stamford, CT). Epidermal growth factor (EGF), AG1478 and anti-β-actin monoclonal antibody were obtained from Sigma (St. Louis, MO).

Plasmids Construction Transcription factor binding sites were identified using TFSEARCH. The construction of Cox-2 promoter plasmid, pGL2-Cox-2-1kb-luc, has been described in Materials and Method section of Chapter 2 of this dissertation. The C/EBP responsive luciferase vector (pGL2-5xCEBP-TKLuc) was generated by cloning five repeats of the C/EBP consensus sequence (TTGCGCAATCT) into the Nhe1 and Hind III sites in front of the herpes simplex virus thymidine kinase (TK) gene promoter (-35-+50) in the pGL2-TK-Luc vector. The C/EBP-β, liver-enriched transcriptional activator protein 1 (LAP1) and LAP2 expression vectors were kindly provided by Dr. L. Sealy (Vanderbilt University School of Medicine) [139, 201]. The expression of C/EBP-β from these vectors in transfected cells was confirmed by immunoblotting. The dominant negative form of C/EBP-β, LIP (liver-enriched inhibitory protein) [139] was cloned into pcDNA3.1 by RT-PCR amplification of a 444 bp cDNA fragment of C/EBP-β from Caov-3 cells. The

structure of pcDNA3-LIP was confirmed by automatic sequencing and immunoblotting analysis of expression of the short, truncated form of C/EBPβ (21 kD) [139]in transfected cells.

Cell Culture The sources of ovarian cancer cell lines used in the study were described previously [9, 192]. These cells were cultured in RPMI medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. All cell lines were frozen at early passages and used for less than 10 weeks in continuous culture.

Western Blot Cells were lysed in SDS sample buffer or in ice-cold X-100 lysis buffer [1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 100 mM NaF, 10 mM Na PPi, and protease inhibitor cocktail]. Total cellular proteins were resolved by SDSPAGE, transferred to Immun-Blot membrane [poly(vinylidene difluoride)] (BIO-RAD, Hercules, CA), and immunoblotted with antibodies following the protocols of manufacturers. Immunocomplexes were visualized with an enhanced chemiluminescence detection kit from Amersham (Piscataway, NJ) using the horseradish peroxidase–conjugated secondary antibodies (Cell Signaling, Danvers, MA).

Transient transfection and luciferase assays Ovarian cancer cell lines were seeded in 6-well plates and grown to 30-40 % confluence before transfection with the luciferase vectors using Fugene 6 (Roche) or TransIT-TKO (Mirus Bio Corp., Madison, WI)

according to the instructions of the manufacturers. About 48 hours after transfection, the cells were starved for 24-36 hours before stimulation with LPA or vehicle for 6 hours. Cell extracts were prepared and assayed for luciferase activity using the luciferase assay kit from Promega. The luciferase activity was normalized on the basis of the activity of cotransfected β-galactosidase reporter driven by the cytomegalovirus promoter (pCMVβ-gal).

Nuclear and Cytoplasmic Extract Preparation – LPA-stimulated or control cells were washing twice with cold PBS, harvested by scraping with a rubber policeman and centrifuged at 1000 rpm for 3 min. Cell pellets were resuspended in a hypotonic lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40], incubated for 15 min on ice, and centrifuged at 3000 rpm. The supernatants containing cytosolic proteins were collected and stored at -80 ^oC immediately. The nuclei pellets were washed once with the hypotonic lysis buffer, resuspended in hypertonic nuclear lysis buffer (50 mM Tris-HCl(pH 8.3), 0.4M NaCl, 40% glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA) and further incubated for 10 mins before centrifugation at 13,000 rpm. The supernatant was collected and quick-frozen in liquid nitrogen before storage in -80 ^oC. Protein concentration was determined with Pierce BCA kit.

Non-radioactive electromobility shift assay (EMSA)- Biotin-labeled self complementary C/EBP consensus oligonucleotides 5’ [biotin] GGTGCAGATTGCGCAATCTGCA 3’ was synthesized by Operon Biotechnologies and annealed in 20 mM Tris (pH 7.4), 1 mM

dithiothreitol, 50 mM NaCl and 10 mM $MgCl_2$. For the gel shift assay, binding reaction was performed by incubating 4 μg of nuclear protein in gel shift buffer (10 mM HEPES (pH 7.8), 10% glycerol, 1 mM EDTA, 25 mM $MgCl_2$, 50 mM KCl, 1 μg of poly(dI.dC), 3 μg BSA and protease inhibitors) in a final volume of 20 μL for 10 mins at 25°C. The biotin-labeled and unlabeled (cold) oligonucleotides were added to the reaction mixture, incubated for an additional 15 mins at 25°C. In supershift experiments, nuclear extracts were incubated for 15 min at room temperature with 1 μg of anti-CEBP-β monoclonal antibody (Santa Cruz Biotech) prior to gel shift reaction. Complexes were separated by electrophoresis on 5% non-denaturing polyacrylamide (PAGE) gel, transferred to a nylon membrane (Amersham Hy-bond XL) and crosslinked using UV Stratalinker 2400 (Stratagene). The signals were visualized by using Phototope-Star biotin detection kit (NEB), according to the manufacturer's protocol.

ChIP assay- Cells were fixed by cross-linking the chromatin with 1% formaldehyde for 5 min, cells, scraped with a rubber policeman and collected by centrifugation. The cells were sonicated six times, 15 seconds each with 1-min intervals under ice by using a Sonic Dismembrator (Fisher Scientific). The average fragment size was 600 bp under these conditions. An equal amount of chromatin was incubated with at least 5 μg of either C/EBP-β-specific immunoglobulin G (IgG) or normal mouse IgG (Santa Cruz Biotech) at 4 °C overnight. IP products were collected after incubation with protein G-coated sepharose beads (Amersham). The beads were washed, protein-DNA cross-links were reversed, and DNA was purified by Qiagen PCR purification columns according to manufacturer's

instructions. Purified DNA from the input and IP samples were subjected to real-time quantitative PCR with uPA promoter specific primers.

ELISA Assay- An enzyme-linked immunosorbent assay (ELISA) was used for the quantification of IL-6. Briefly, culture supernatants of Caov-3 treated without or with LPA or other ligands were collected and analyzed for IL-6 using the Quantikine IL-6 ELISA kits (R&D Systems) according the manufacturer's instruction. Colorimetric reactions were read on a EL800 microplate reader (Bio-TEK Instruments, Winooski,VT). The concentrations of IL-6 in samples were calculated by comparing with those of standards provided with the ELISA kits.

Stable expression of C/EBP-β LIP by retrovirus – The human LIP cDNA was excised from pcDNA3-LIP using HindIII and EcoRI and cloned into a moloney murine leukemia retrovirus vector, S-001AB (LZRS-EGFP)(a gift from Dr. J. Chun, the Scripps Research Institute, La Jolla, CA). The complete insert and flanking regions of the final retrovirus vector were confirmed by automatic sequencing. The LZRS-EGFP-LIP and control vector were transfected into the PZ67 packaging cell line with lipofectamine 2000 as previously described [9]. Virus stocks were harvested 72 hrs post-transfection by centrifugation of the supernatants at 2100 rpm for 10 min and stored in -80 °C. Skov-3 cells grown in 6-well dishes were infected with 1.4 ml virus to cells in the presence of 7.5 μg/ml polybrene (Sigma-Aldrich) and incubated at 37 ^{o}C overnight. The infection medium was then replaced with growth medium. Cells were split into 10 cm dishes after 48 hours. Viral-

transduced GFP-positive cell were sorted out by flow cytometry at the VCU flow cytometry core facilities.

Table 3.1 Plasmids and Primers

Plasmid	Wildtype or Mutation primers
pGL2-Cox-1kb-Luc	5'-TTTAGCGTCCCTGCAAATTCTGGC-3'(SENSE) 5'-CGCTCACTGCAAGTCGTATGACAA-3' (ANTISENSE)
pGL2-IL-6-Luc	5'- GAGCTCAAGGATCCTCCTGCAAGAGA -3'(SENSE) 5'- TAGAGCTTCTCTTTCGTTCCCGGTGG -3' (ANTISENSE)
pGL2-IL-6-mut-Luc	5'- GGACGTCACAGTCTACTCTCTTAATAAGGTTTCC -3'(SENSE) 5'- GGAAACCTTATTAAGAGAGTAGACTGTGACGTCC -3' (ANTISENSE)
pGL2-uPA-Luc	5'- CGGATCACAAGGTCAGGAAGATCGAG -3'(SENSE) 5'- TCTCCGACTGTGCTGCGA C 3' (ANTISENSE)
pGL2-uPA-mut-Luc	5'- GAGGCAGTCTTAGGCGGGTTGGGGCCCAGCG -3'(SENSE) 5'- CGCTGGGCCCCAACCCGCCTAAGACTGCCTC -3' (ANTISENSE
pGL2-5xCEBP-TK-Luc	5'CTAGCATCTAAGTTTGCGCAATCTTTGCGCAATCTTTGCGCAATCTTTGC GCAATCTTTGCGCAA -3'(SENSE) 5'AGCTTTTGCGCAAAGATTGCGCAAAGATTGCGCAAAGATTGCGCAAAGA TTGCGCAAACTTAGAT 3' (ANTISENSE)
pcDNA3-LIP	5'-GGAATCAAGCTTGGCGCACATGGCGGCG-3' (SENSE) 5'-GCAATACTCGAGCGCTAGCAGTGGCCGGAGG-3' (ANTISENSE).

3.3 Results

3.3.1 LPA induces phosphorylation and protein expression of C/EBP-β

We previously established the necessity for C/EBP binding and transcriptional activity in LPA-induced gene expression using Cox-2 as a model [232]. A major biochemical process associated with transcriptional activation of C/EBP-β is phosphorylation. We examined whether LPA triggers activation of C/EBP-β by

modulating the phosphorylation status of the protein. By western blot analysis of total cell extract from Caov-3 cells, we showed that stimulation with LPA caused a rapid phosphorylation of C/EBP-β (Fig. 3.1 A). The signal decreased after 30 mins incubation with LPA. However, levels of phosphorylated C/EBP-β remained substantially elevated above background hours after exposure to LPA. In some cell models, phosphorylation has been shown to be prerequisite for nuclear transportation of C/EBP-β [142]. As such, we assessed the influence of LPA stimulation on nuclear-cytoplasmic partitioning of C/EBP-β. As shown in Fig. 3.1 B and Fig. 3.1 C, C/EBP-β was exclusively resident in the nucleus in both untreated and treated Caov-3 and Skov-3 cells. Further, LPA did not induce cytoplasmic translocation of C/EBP-β, an event that has been shown to result in suppression of C/EBP-β target genes [233, 234]. However, we observed significant increase in expression levels of C/EBP-β after prolonged stimulation of ovarian cancer cells with LPA (Fig. 3.1 B and C). This is consistent with the fact that C/EBP-β promoter possesses binding sites for C/EBP [235, 236]. Thus, C/EBP-β may be auto-induced in LPA-treated cells.

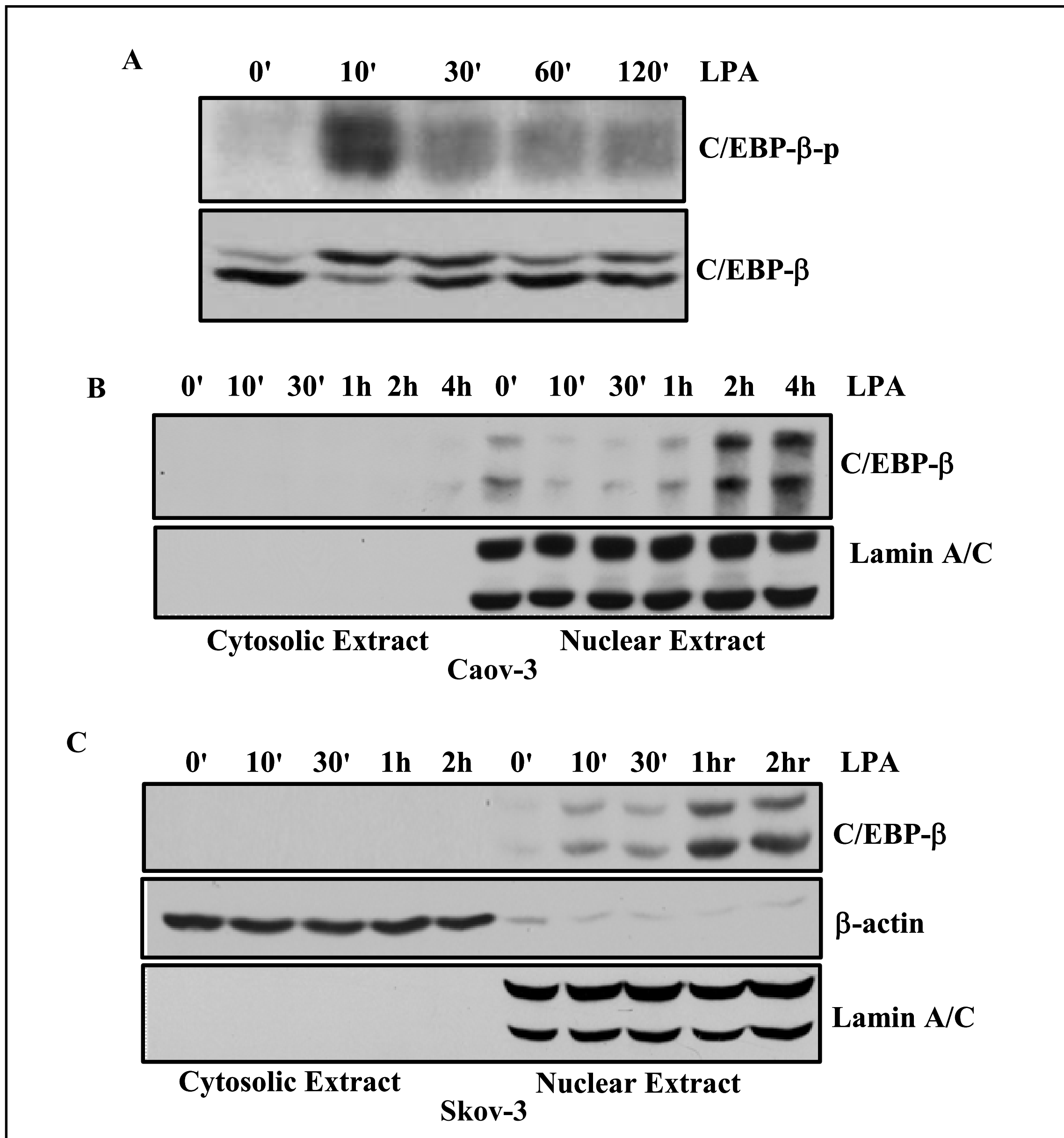


Fig. 3.1 LPA induces phosphorylation and expression of C/EBP-β in ovarian cancer cells.
A. Caov-3 cells were stimulated with 10 μM LPA for the indicated periods of time. Total cell lysates were analyzed by western blotting for phosphorylated C/EBP-β. In **B** and **C**, C/EBP-β protein levels were analyzed by western blotting in cellular fractions of serum-starved Caov-3 and Skov-3 cells respectively, following treatment with 10 μM of LPA for indicated periods of time. β-actin was used as loading control for cytoplasmic protein and lamin a/c as control for nuclear proteins.

Pretreatment of Caov-3 cells with Cycloheximide, an inhibitor of protein synthesis, completely abrogated LPA-induced C/EBP-β expression (Fig. 3.2). This confirmed that the increase in C/EBP-β at later time points was indeed a consequence of new protein synthesis. It is likely that newly synthesized C/EBP-β may be further activated in the presence of LPA. The ability of LPA to elevate C/EBP-β in the nucleus may be part of a mechanism to ensure the long term effects of LPA on gene expression.

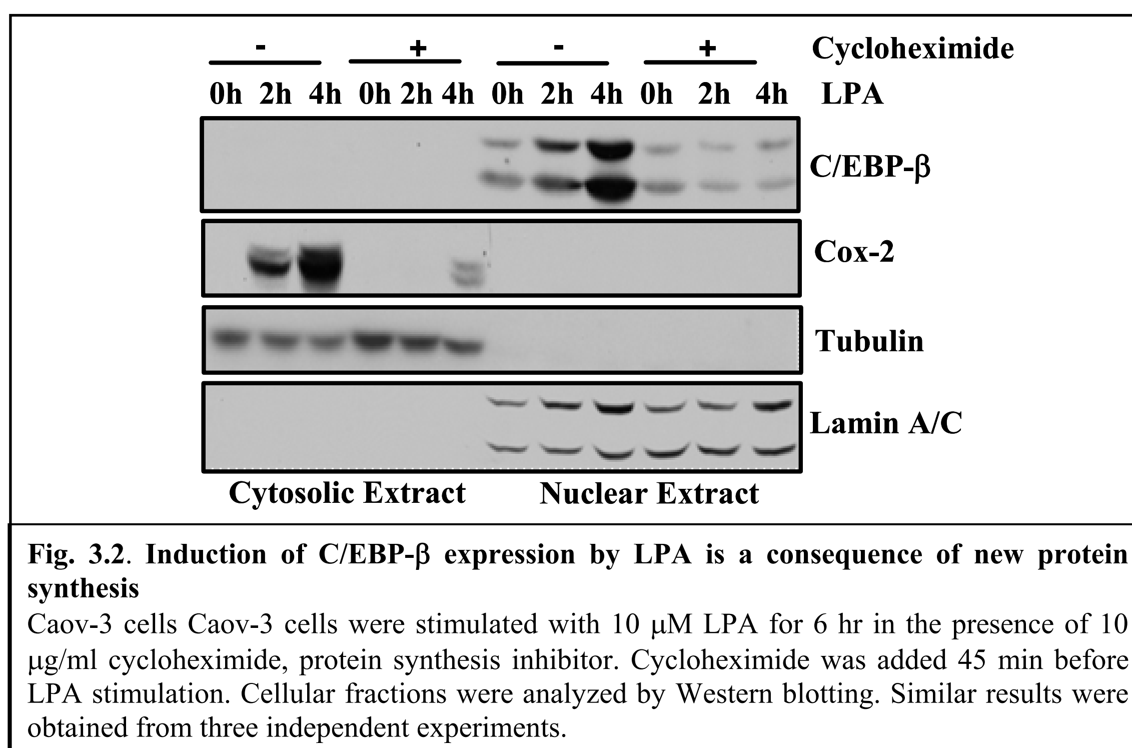


Fig. 3.2. Induction of C/EBP-β expression by LPA is a consequence of new protein synthesis
Caov-3 cells Caov-3 cells were stimulated with 10 μM LPA for 6 hr in the presence of 10 μg/ml cycloheximide, protein synthesis inhibitor. Cycloheximide was added 45 min before LPA stimulation. Cellular fractions were analyzed by Western blotting. Similar results were obtained from three independent experiments.

3.3.2 LPA induces C/EBP-β DNA-binding and transcriptional activities

C/EBP-β contains intramolecular inhibitory elements that hinder its DNA binding site [237]. Phosphorylation of C/EBP-β has been shown to increase its DNA binding activity in vitro [220]. To determine whether C/EBP-β phosphorylation induced by LPA result in enhancement in C/EBP-β DNA binding activity, we performed electromobility shift assay. The binding of C/EBP to consensus oligonucleotides was increased following LPA stimulation (Fig 3.3 A) and bound complexes were supershifted in the presence of anti-C/EBP-β. The binding depended on C/EBP-β phosphorylation as it was blocked by pretreatment of nuclear extract with potato alkaline phosphatase (PAP) to remove phosphate groups (Fig 3.3 B). Further, C/EBP-DNA complexes significantly increased at later time points when more C/EBP-β was present (Fig. 3.3 A and B).

Next, we constructed a C/EBP-responsive luciferase plasmid (pGL2-5xCEBP-TK-Luc), in which five copies of the C/EBP consensus sequence were linked to the basic TK promoter. As shown by Fig. 3.3 C, LPA stimulated 5-10 fold increase in luciferase activity in pGL2-5xCEBP-TK-Luc-transfected ovarian cancer cells but not in the cells transfected with the backbone vector lacking the C/EBP responsive sites (pGL2-TK-Luc). Taken together, these results indicate that LPA induces C/EBP-β DNA-binding and transcriptional activities.

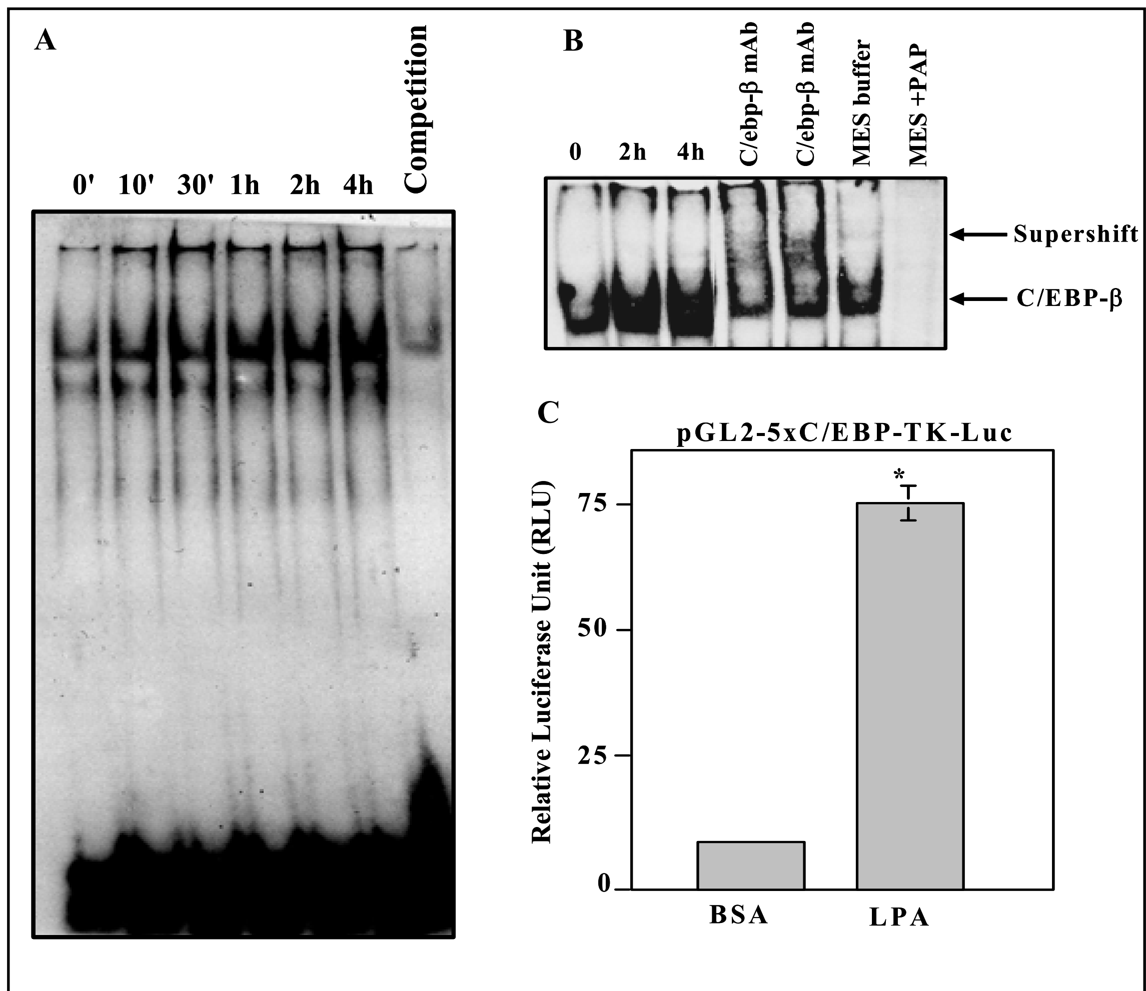


Fig. 3.3. **LPA activates binding and transcriptional activities of C/EBP-β .**
A, Nuclear extracts obtained from Caov-3 cells stimulated with 10 μM LPA for the indicated periods of time were subjected to gel shift assay (EMSA) (see Materials and Methods). Supershift experiments were performed on 2h LPA-treated nuclear extracts using 2 and 5 μg anti-C/EBP-β mouse monoclonal antibody. For dephosphorylation of nuclear extracts, 1 unit of potato alkaline phosphatase (PAP) was added for 15 min prior to binding reaction (**B**). In **C**, Caov-3 cells were transfected with a luciferase construct of a 5 copies of C/EBP binding sites upstream of a TK promoter. Cells were treated with 10 μM LPA for 6 hours and analyzed for luciferase activity.

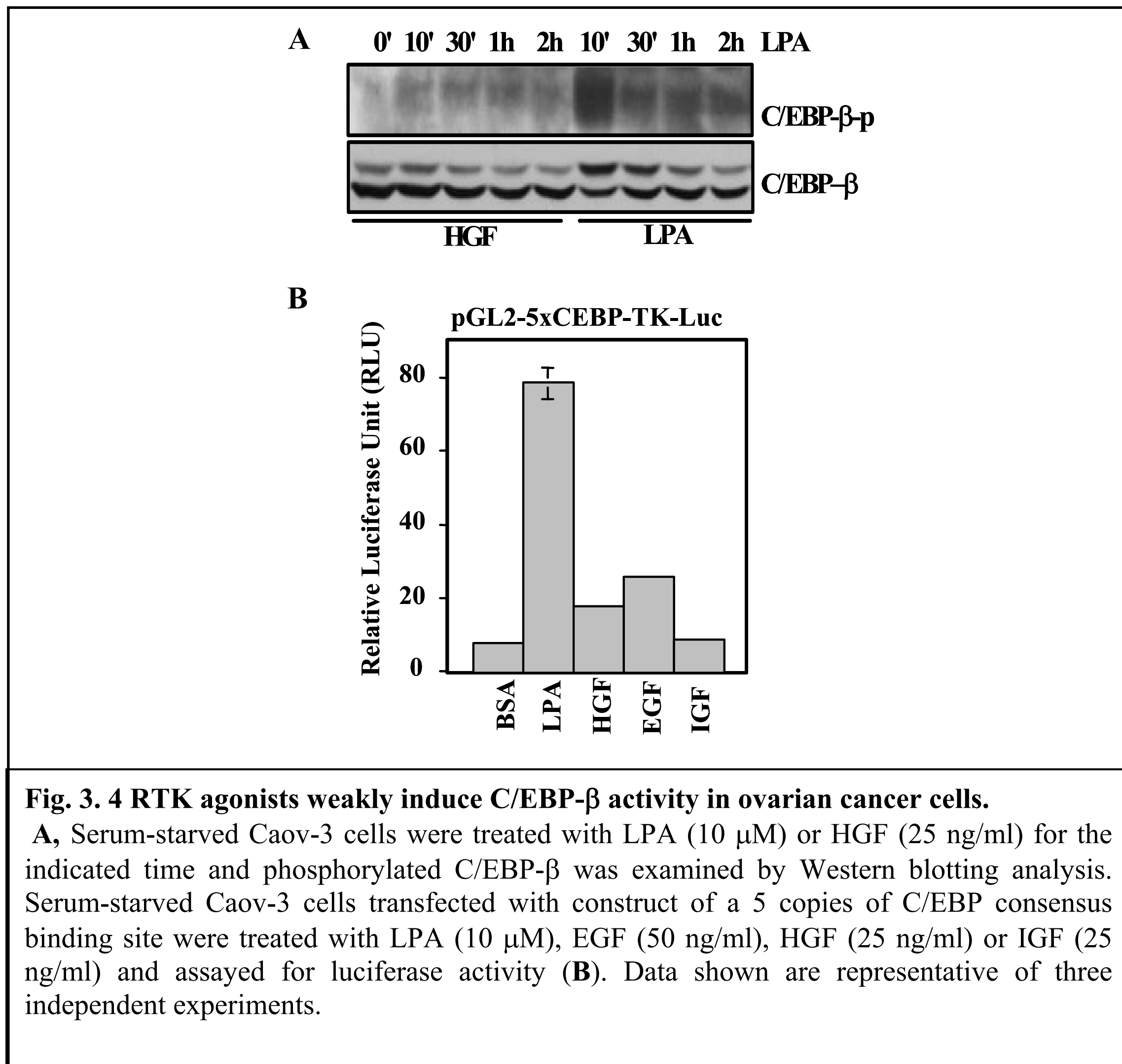


Fig. 3. 4 RTK agonists weakly induce C/EBP-β activity in ovarian cancer cells.
A, Serum-starved Caov-3 cells were treated with LPA (10 μM) or HGF (25 ng/ml) for the indicated time and phosphorylated C/EBP-β was examined by Western blotting analysis. Serum-starved Caov-3 cells transfected with construct of a 5 copies of C/EBP consensus binding site were treated with LPA (10 μM), EGF (50 ng/ml), HGF (25 ng/ml) or IGF (25 ng/ml) and assayed for luciferase activity (**B**). Data shown are representative of three independent experiments.

3.3.3 LPA stimulates activation of C/EBP-β through a regulatory mechanism integrating GPCR signal(s) and a permissive activity of RTK

In comparison with LPA, agonists of RTKs including EGF, HGF and IGF only weakly induced the expression of LPA-target genes such as Cox-2 (Fig. 2.2 D). Similarly, C/EBP-β seemed to be preferentially activated by LPA because RTK agonists, EGF, IGF

and HGF, were weak stimuli of C/EBP-β phosphorylation (Fig. 3.4 A) and transcriptional activity (Fig. 3.4 B). However, pretreatment of Caov-3 cells with a specific inhibitor of EGFR kinase activity, AG1478, resulted in a profound decrease in LPA-induced phosphorylation of C/EBP-β (Fig. 3.5 A). AG1478 attenuated the LPA-induced increase in C/EBP-β protein levels (Fig. 3.5 B). In addition, LPA-induced binding of C/EBP to its consensus oligonucleotide (Fig. 3.5 C) and C/EBP mediated transactivation, as measured by the C/EBP-responsive luciferase vector were significantly inhibited by AG1478 (Fig. 3.5 D). Therefore, LPA-induced C/EBP-β phosphorylation, DNA binding and functional activation were invariably sensitive to EGFR inhibition suggesting that EGFR activity, albeit insufficient on its own to trigger strong C/EBP-β activation, was required for LPA GPCR signaling to C/EBP-β.

We observed that compared to EGF, LPA only weakly induced activation of EGFR, as assessed by phosphorylation at Y-1068 (Fig. 3.5 E), ruling out the possibility that LPA activation of C/EBP-β was through transactivation of EGFR. To further explore this novel mode of crosstalk between the two receptor types, we examined whether the requirement of EGFR in the process could be relieved by activation of another RTK, such as c-Met. Treatment of Caov-3 cells with HGF alone only slightly activated C/EBP-β phosphorylation (Fig. 3.4 A) and C/EBP transcriptional activity (Fig. 3.4 B). However, when EGFR was inhibited by AG1478, LPA was fully capable of stimulating C/EBP-β phosphorylation (Fig. 3.5 A) and transcriptional activity (Fig. 3.5 D) if cells were co-stimulated with HGF to activate c-Met. Taken together, these results demonstrate that

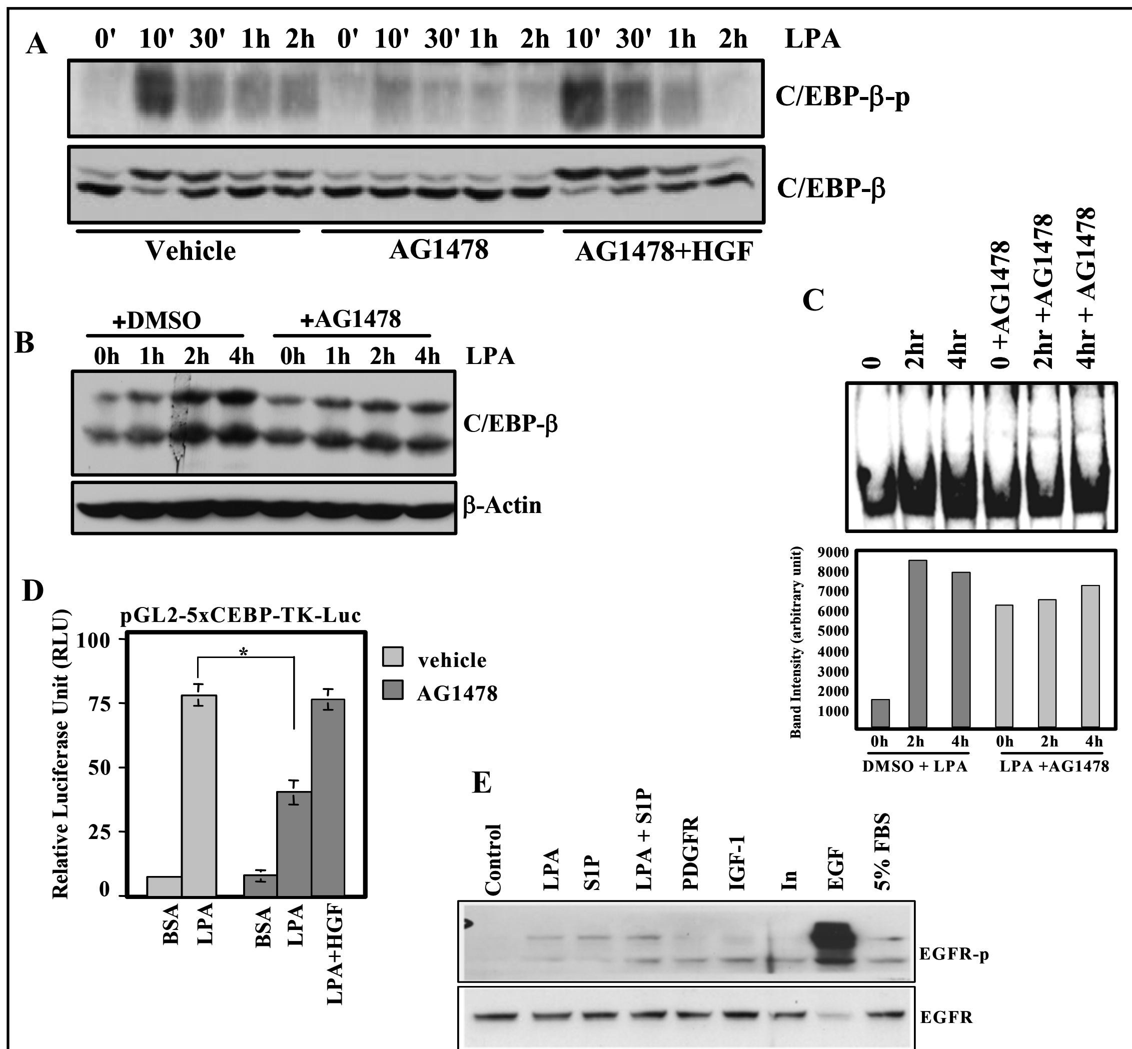


Fig. 3.5. **EGFR inhibitor blocks LPA-induced activation of C/EBP-β**
Serum-starved Caov-3 cells were treated with LPA (10 μM) and/or AG1478 (1 μM) for the indicated time and phosphorylated (**A**) or total C/EBP-β (**B**) was examined by Western blotting analysis. Co-stimulation with HGF (25 ng/ml) in the presence of AG1478 restored LPA-induced phosphorylation of C/EBP-β (**A**). **C**, nuclear extracts from LPA-treated or untreated Caov-3 cells were analyzed by gel shift assay using biotin-labeled consensus C/EBP oligonucleotides (see Materials and Methods). Band intensities were quantified with NIH Image J tool. In **D**, Caov-3 cells transfected with a luciferase construct of a 5 copies of C/EBP binding sites upstream of a TK promoter. Serum-starved transfected cells were then pretreated AG1478, stimulated with LPA and/or HGF for 6 hours and examined for luciferase activity. Cell lysates of Caov-3 cells stimulated indicated agonists for 30' were analyzed by western blotting for tyrosine 1068 phosphorylation of EGFR (**E**). Results are representative of three independent experiments.

RTK, not necessarily EGFR, provides an obligatory activity that acts in concert with LPA GPCR signaling to activate C/EBP-β.

3.3.4 RTK-dependent activation of C/EBP-β mediates induction of Cox-2 and other LPA-target genes

Our observation that LPA induced the expression of Cox-2 in a C/EBP-β-dependent manner led us to ask whether the requirement of RTK for C/EBP-β activation also applied to LPA-induced Cox-2 gene expression. Compared to LPA, agonists of RTK, including EGF, HGF and IGF only weakly induced Cox-2 expression in Caov-3 cells as shown in Fig. 3.6 A. Similarly, these RTK agonists were weak stimuli of C/EBP-β transcriptional activity as indicated by luciferase assays with the C/EBP-responsive construct pGL2-5x-CEBP-TK-luc (Fig. 3.4 B). Thus, C/EBP-β seems to be rate-limiting regulator of Cox-2, preferentially activated by GPCRs rather than RTKs. In further support of the role of C/EBP-β in the transcriptional activation of Cox-2, HGF efficiently reversed the inhibitory effect of AG1478 not only on C/EBP-β activation (Fig. 3.5 A and D) but also on LPA-induced Cox-2 (Fig. 3.6 B).

In addition to Cox-2, a subset of LPA target genes including IL-6 and uPA bear binding sites for C/EBP in their promoters (Fig. 1.2 of Chapter 1). IL-6 is a pleiotropic cytokine and a prominent mediator of inflammation. ELISA analyses of conditioned medium from Caov-3 cells treated with LPA showed a robust increase in IL-6 production (Fig. 3.6 C). The ability of LPA to induce the expression of IL-6 in many cell models may

explain why high concentrations of IL-6 are present in the ascites of ovarian cancer patients [238, 239]. In addition, IL-6 levels in ovarian cancer correlate with poor prognosis [240].

It has been reported that C/EBP-β activates IL-6 promoter in response to IL-1, bacterial LPS and many other stimuli [241, 242]. To assess the contribution of C/EBP-β to LPA-induced IL-6 expression, we constructed a luciferase reporter carrying 1.2 kb of wildtype IL-6 promoter. Disruption C/EBP binding site by point mutation dramactically decreased the IL-6 promoter activity induced by LPA by 60% loss in promoter response to LPA as measured by luciferase assay (Fig 3.6 D). Co-transfection of Caov-3 cells with inhibitory C/EBP-β isoform, LIP, also attenuated the IL-6 promoter activation by LPA (Fig. 3.6 E). Stable expression of LIP in Skov-3 cells (Fig. 3.6 F, *lower right*) (see Materials and Methods) caused a modest reduction of LPA-induced IL-6 production (Fig. 3.6 F), suggesting that stably-transfected Skov-3-LIP cells may have activated some mechanisms to suppress the effects of enhanced LIP/LAP ratio, such that the impact of inhibiting transcriptional activities of C/EBP-β on LPA-induced IL-6 gene expression becomes not as dramatic as in transient experiments. However, AG1478 inhibited LPA-induced IL-6 promoter response and IL-6 production (Fig. 3.6 G). Similarly, co-stimulation with HGF restored the ability of LPA-stimulated Caov-3 cells to generate IL-6.

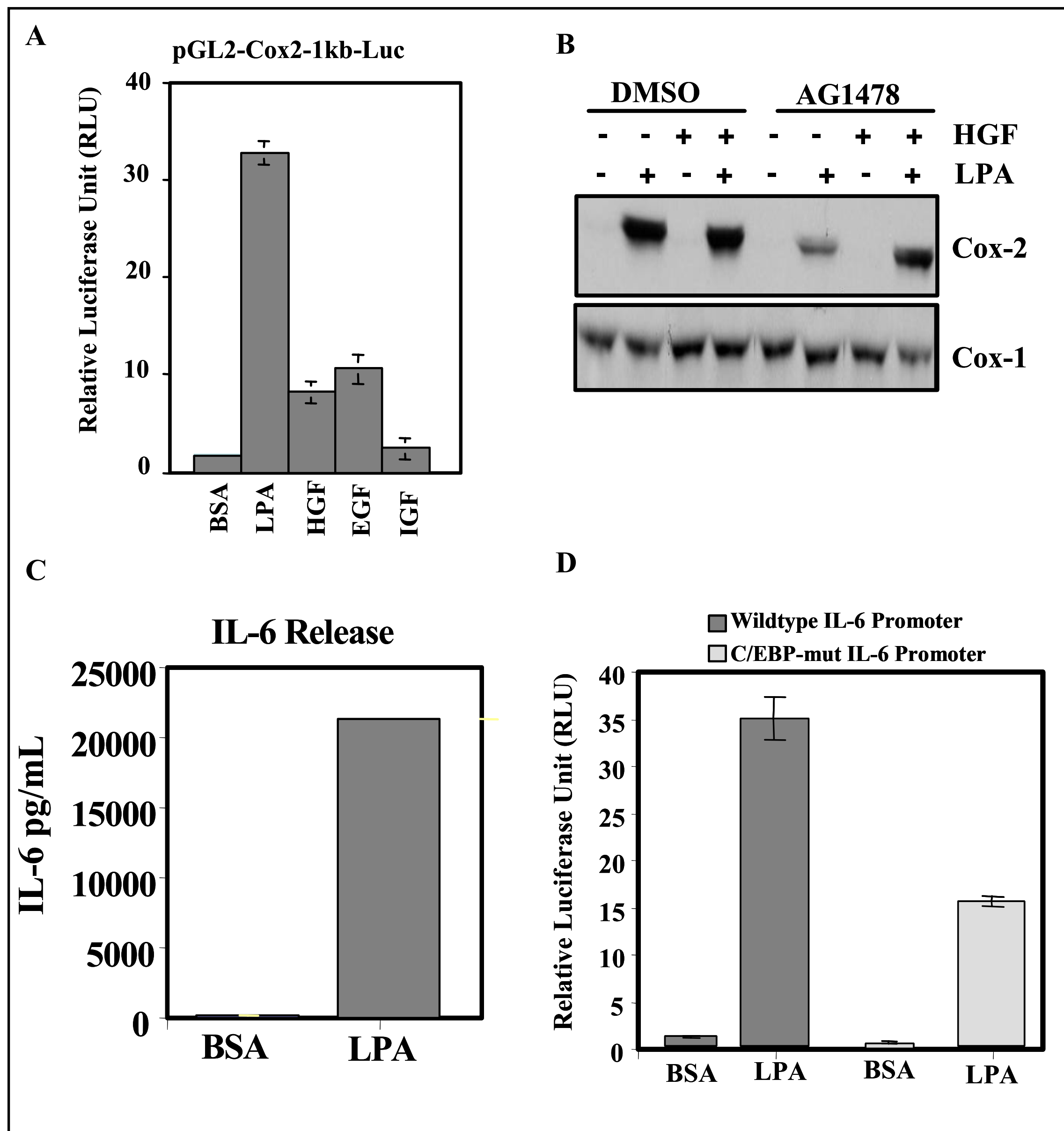


Fig. 3.6. **RTK-dependent activation of C/EBP-β mediates induction of Cox-2 and IL-6.** ***A*****.** Serum-starved Caov-3 cells transfected with Cox-2 promoter construct were treated with LPA (10 μM), EGF (50 ng/ml), HGF (25 ng/ml) or IGF (25 ng/ml) and assayed for luciferase activity (**B**) Cox-2 protein levels in Caov-3 cells pretreated AG1478 and stimulated with LPA and/or HGF, were examined by Western blotting analysis. In **C**, cultured supernatant of Caov-3 cells treated with or without LPA for 18hrs were assayed for IL-6 by ELISA. **D**, Caov-3 cells transfected with wildtype or C/EBP mutated IL-6 promoter were treated with LPA for 6 hours before luciferase assay.

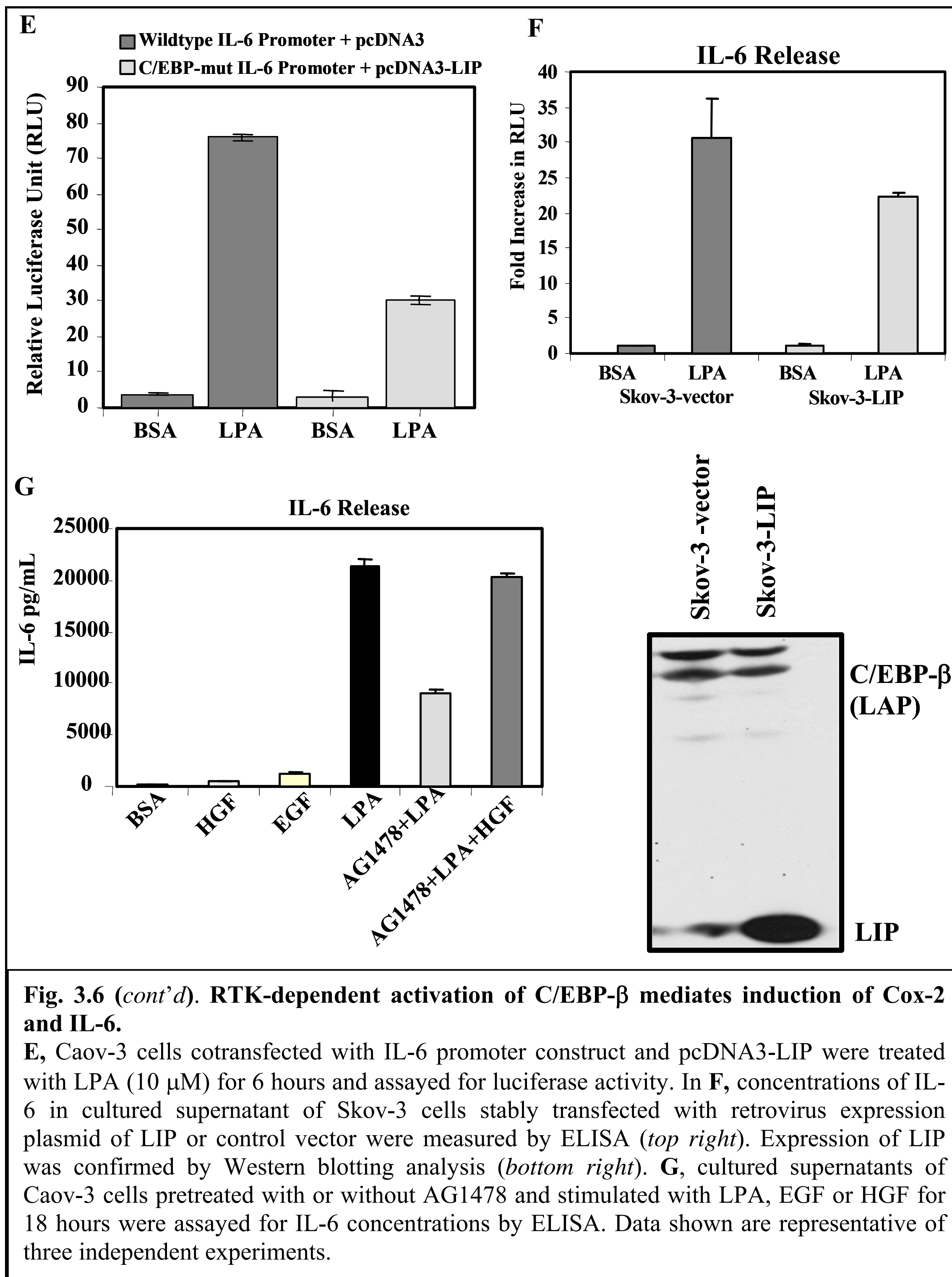


Fig. 3.6 (*cont'd*). RTK-dependent activation of C/EBP-β mediates induction of Cox-2 and IL-6.

E, Caov-3 cells cotransfected with IL-6 promoter construct and pcDNA3-LIP were treated with LPA (10 μM) for 6 hours and assayed for luciferase activity. In **F,** concentrations of IL-6 in cultured supernatant of Skov-3 cells stably transfected with retrovirus expression plasmid of LIP or control vector were measured by ELISA (*top right*). Expression of LIP was confirmed by Western blotting analysis (*bottom right*). **G**, cultured supernatants of Caov-3 cells pretreated with or without AG1478 and stimulated with LPA, EGF or HGF for 18 hours were assayed for IL-6 concentrations by ELISA. Data shown are representative of three independent experiments.

Finally, consistent with previous studies, LPA induced the activation of uPA, an important component of tumor cell invasion and metastasis (Fig. 3.7 A). We evaluated the involvement of C/EBP-β in LPA-induced activation of uPA gene expression. By utilizing ChIP assay (see Materials and Methods), we found an increased presence of C/EBP-β at the uPA promoter after 2 hours following LPA stimulation (Fig. 3.7 B). Compared to wildtype construct, mutation of the proximal C/EBP binding site on uPA promoter (uPAmut-luc) resulted in a partial yet significant decrease in LPA-induced promoter activity (Fig. 3.7 C). Co-transfection of LIP in Caov-3 cells also markedly decreased uPA promoter activation by LPA (Fig. 3.7 D). In agreement with activation of C/EBP-β, AG1478 completely abrogated LPA-induced uPA activation (Fig. 3.7 E). These results established that RTK-dependent activation of C/EBP-β reflects a general signaling mechanism to regulate expression of LPA-target genes Cox-2, IL-6 and uPA.

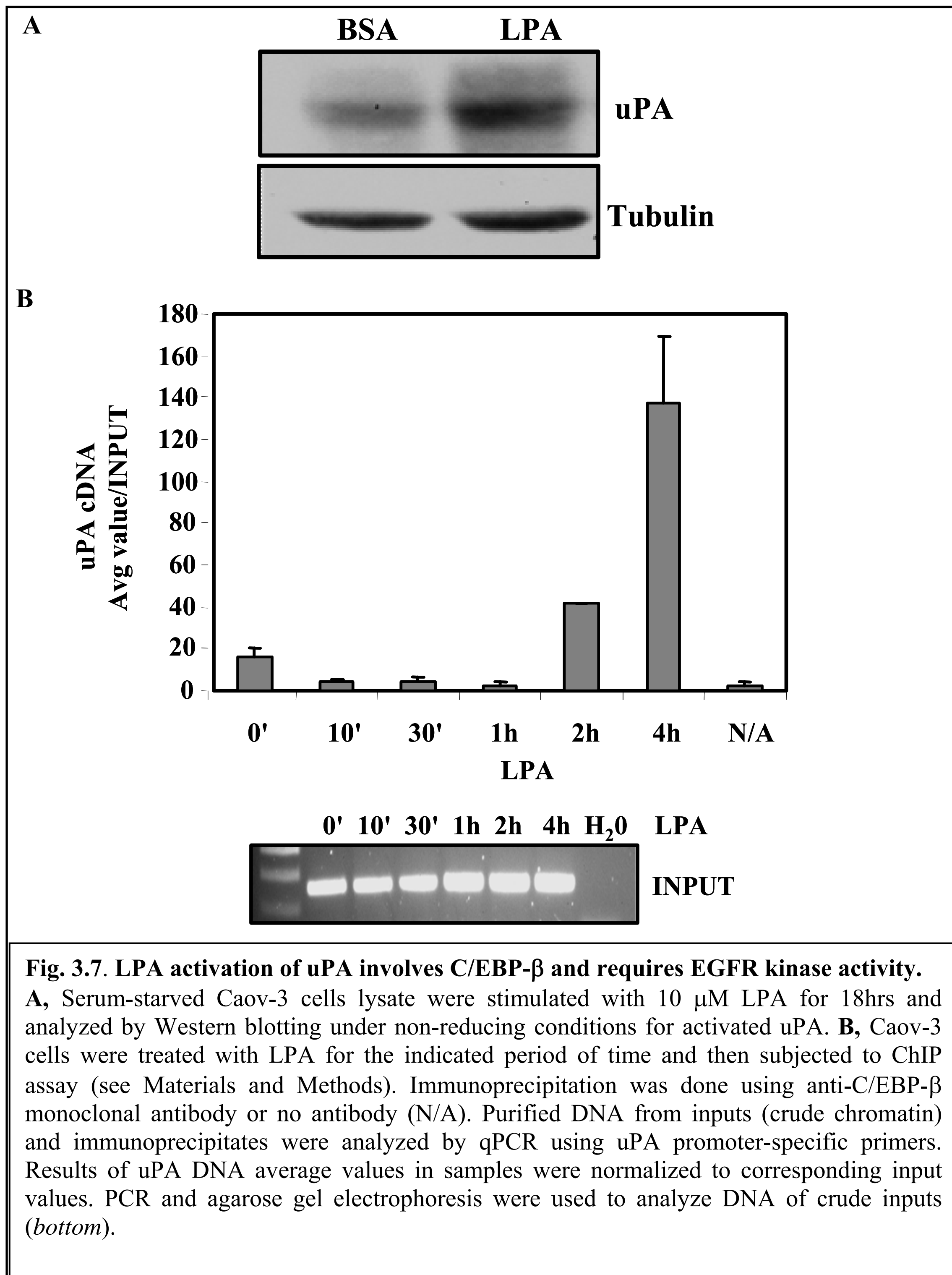


Fig. 3.7. **LPA activation of uPA involves C/EBP-β and requires EGFR kinase activity.** **A,** Serum-starved Caov-3 cells lysate were stimulated with 10 μM LPA for 18hrs and analyzed by Western blotting under non-reducing conditions for activated uPA. **B,** Caov-3 cells were treated with LPA for the indicated period of time and then subjected to ChIP assay (see Materials and Methods). Immunoprecipitation was done using anti-C/EBP-β monoclonal antibody or no antibody (N/A). Purified DNA from inputs (crude chromatin) and immunoprecipitates were analyzed by qPCR using uPA promoter-specific primers. Results of uPA DNA average values in samples were normalized to corresponding input values. PCR and agarose gel electrophoresis were used to analyze DNA of crude inputs (*bottom*).

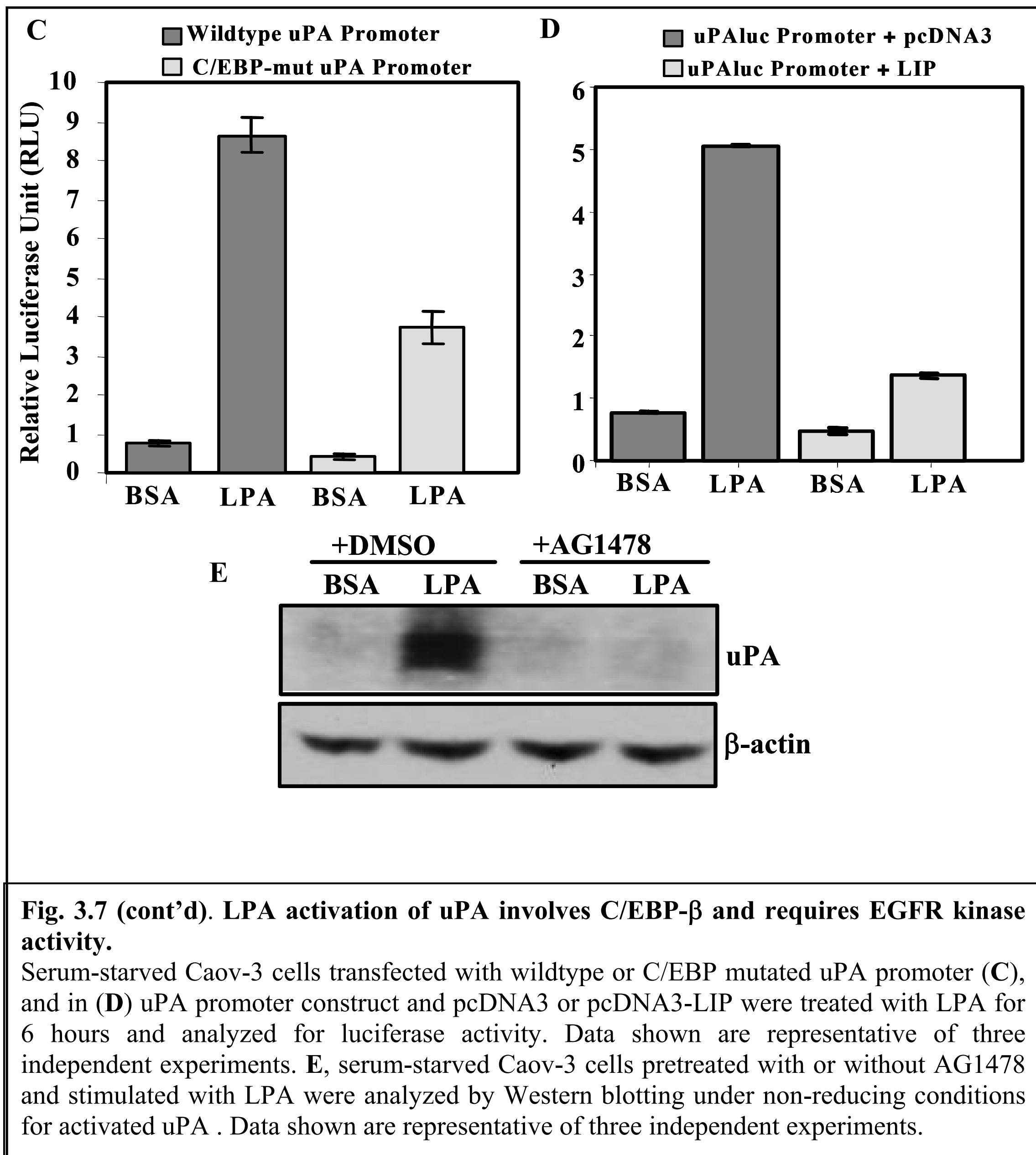


Fig. 3.7 (cont'd). **LPA activation of uPA involves C/EBP-β and requires EGFR kinase activity.**

Serum-starved Caov-3 cells transfected with wildtype or C/EBP mutated uPA promoter (**C**), and in (**D**) uPA promoter construct and pcDNA3 or pcDNA3-LIP were treated with LPA for 6 hours and analyzed for luciferase activity. Data shown are representative of three independent experiments. **E**, serum-starved Caov-3 cells pretreated with or without AG1478 and stimulated with LPA were analyzed by Western blotting under non-reducing conditions for activated uPA . Data shown are representative of three independent experiments.

3.4 Discussion

In this chapter, we explored the signaling mechanisms by which LPA stimulates activation of C/EBP-β, a key transcription factor in LPA induction of Cox-2. Our results indicated that LPA induced phosphorylation of C/EBP-β, which correlated with increase in C/EBP-β DNA binding activity. By analyzing different cellular fractions, we found that C/EBP-β was essentially localized to the nuclei in ovarian cancer cells. A very minute amount was present in the cytoplasmic fraction. LPA did not cause any translocation of C/EBP-β between cytosol and nucleus. At later hours, there was net increase in total C/EBP-β protein levels in LPA-treasted cells, probably reflecting auto-induction by C/EBP-β itself as the C/EBP-β promoter contains C/EBP consensus sequences. The increase in C/EBP-β protein levels could contribute to sustained effects of LPA on expression of Cox-2 and other LPA-target genes.

In pursuit of the molecular mechanism linking the LPA receptors to C/EBP-β activation, we observed that LPA-induced C/EBP activation involves an obligatory activity from EGFR. Fig. 3.8 is a simplified representation of this obligatory role of EGFR in LPA GPCRs signaling. Transactivation of EGFR has been proposed as a mechanism to mediate many biological actions of LPA in numerous studies [181, 243]. In contrast, the possibility for involvement of a parallel RTK signal in biological responses to LPA or other GPCR agonists has been rarely studied [189]. In our experiments, EGF and other RTK agonists only weakly stimulated C/EBP-β activity compared to LPA, allowing us to distinguish the input of GPCR from that of RTK in these cells.

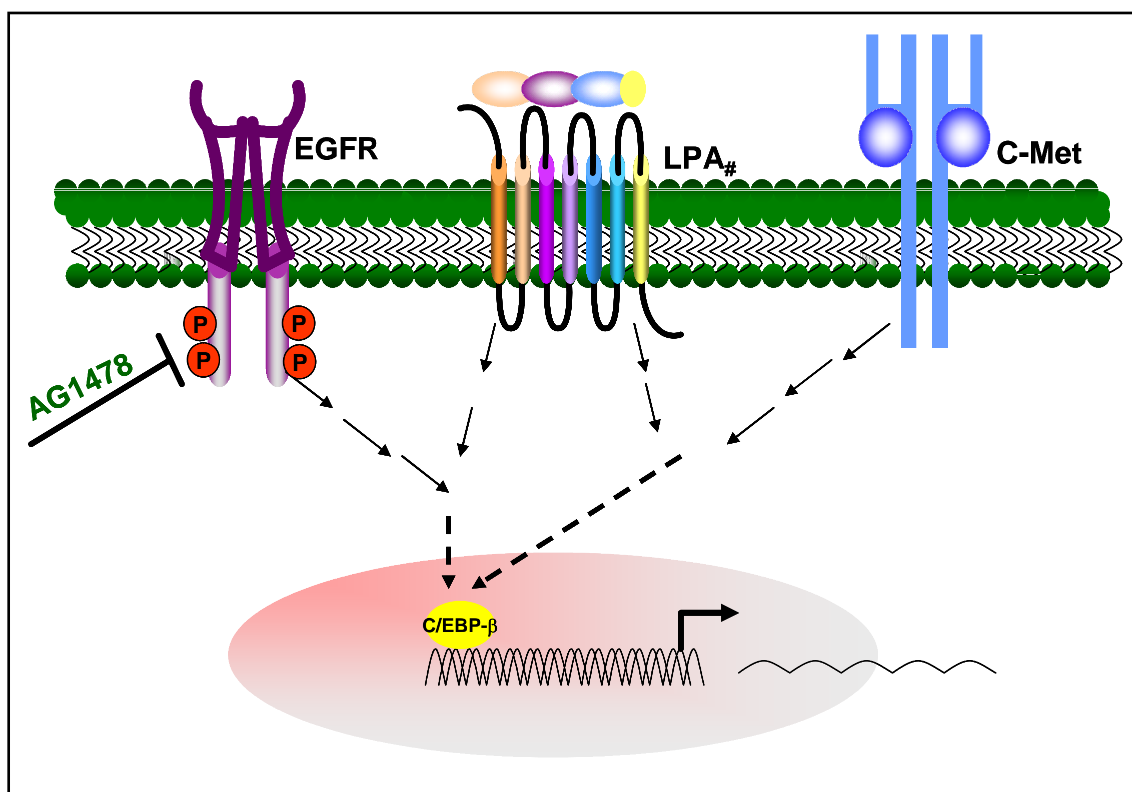


Fig. 3.8. Hypothetical model of LPA GPCR-RTK crosstalk in activation of transcription factors and gene expression.
Basal activity of EGFR synergizes with signals from LPA GPCR to mediate transcription factor activation. Inhibition of EGFR kinase activity with pharmacological inhibitor AG1478 attenuates LPA-induced activation of gene expression. Activation of another RTK, c-Met by costimulation with HGF in the presence of AG1478 rescues LPA signals to transcription factors.

Based on the differential abilities of LPA and EGF to activate C/EBP-β, it is hard to imagine that transactivation of EGFR could trigger robust activation of C/EBP-β and Cox-2 expression in LPA-stimulated cells. It is more likely that optimal activation of C/EBP-β relies on combinatorial signaling components from LPA GPCR(s) and EGFR. The EGFR signal may feed in at some point downstream of GPCRs. Furthermore, our

results indicate that activities of other RTKs, not necessarily EGFR, could cooperate with the LPA GPCR(s) in regulation of C/EBP activity. EGFR is usually recognized to serve such a role probably because it is more universally expressed and exhibits higher activity than other RTKs, particularly in cancer cells [244]. The high EGFR activity present in malignant cells may be necessary for appropriate GPCR signaling.

In addition to Cox-2, we demonstrated that two other LPA-target genes, IL-6 and uPA are also induced at least partially through activation of C/EBP-β. Thus, the effects of LPA on IL-6 production and uPA activation were sensitive to inhibition of EGFR. The comprehensive evaluation of C/EBP-β activation in regulation of gene expression by LPA described in the present study has further implicated this transcription factor in two important parts of tumor progression: inflammation and metastasis. Early immediate response genes such as Cox-2 require prompt transcription. The rapid induction of C/EBP-β presence to Cox-2 promoter upon LPA stimulation (Fig. 2.12 of Chapter 2) indicate that C/EBP-β may play critical roles in the initiation of transcription and the recruitment of transcription factors and co-activators to the Cox-2 promoter [123, 143]; thus its activities may not be necessary for continued transcription. Unlike Cox-2, activated uPA, a member of a 3-component metastasis system (others are uPA receptor [uPAR] and plasminogen activator inhibitors 1 and 2 [PAI-1 and PAI-2]), often accumulates in late response to stimulation, a result of dependence on the activation of other early response genes [245-248]. C/EBP-β appeared much later near uPA promoter upon LPA stimulation, clearly demonstrating that continued activation of C/EBP-β significantly contributes to both instantaneous and prolong or sustained induction of LPA-target genes.

CHAPTER 4

DIFFERENTIAL REQUIREMENT OF RECEPTOR TYROSINE KINASE ACTIVITY FOR LYSOPHOSPHATIDIC ACID-INDUCED ACTIVATION OF G PROTEIN SIGNALING CASCADES AND TRANSCRIPTION FACTORS

4.0 Abstract

The role of the epidermal growth factor receptor (EGFR) in provoking biological actions of G protein-coupled receptors (GPCRs) has been one of the most controversial research subjects in the field of GPCR signal transduction. We and others have recently provided evidence for a permissive input from a receptor tyrosine kinase for activation of GPCR signaling [18, 232, 260]. As described in Chapter 3, LPA-induced activation of CEBP-β and CEBP responsive genes (Cox-2 and IL-6) requires a RTK activity. In the current study, we substantiated the crosstalk between the two receptor subtypes to determine where the RTK input is integrated with GPCR signals to stimulate transcription factors. In ovarian cancer cell lines, activation of AP-1 components by LPA also depended upon EGFR, which could be prevented by activation of another receptor tyrosine kinase c-Met with hepatocyte growth factor (HGF), indicating that LPA-induced activation of AP-1 requires a permissive signal from a receptor tyrosine kinase, not necessarily EGFR. In contrast, LPA induced activation of another prominent transcription factor NF-κB in an

EGFR-independent manner. These differential requirements indicate that the RTK activity is involved in activation of selective signaling pathways downstream of LPA receptors rather than activation of LPA receptors themselves. In keeping with this, EGFR was required for LPA-induced activation of G_i, but not G_q or $G_{12/13}$ as determined by analyzing respective effectors of individual classes of G proteins. Further molecular and pharmacological experiments indicated that G_i was essential for activation of AP-1 by LPA while NF-κB activation lied downstream of the EGFR-independent G_q pathway. Consistent with essential roles for G_i and the downstream AP-1 transcription factor in pleiotropic biological processes, most of cellular responses to LPA such as cytokine production, cell proliferation, migration and invasion required intact EGFR. These results reveal a novel dimension of molecular mechanism for the requirement of RTK in transmission of GPCR signals.

4.1 Introduction

The LPA receptors couple to multiple G proteins, $G_{12/13}$, G_i, G_q, and probably G_s [35, 39, 41, 42, 249]. These G proteins link to diverse signaling pathways including stimulation of phospholipase C and D [16, 49], inhibition of adenylyl cyclase [49], and activation of Ras and the downstream mitogen-activated protein kinases and phosphoinositide 3-kinase [50, 52]. Activation of these signaling cascades downstream of LPA receptors culminates in morphological changes and promotion of cell growth, survival and motility [50, 51]. Recently, we and others demonstrated that LPA induces

activation of transcription factors, upregulating expression of many target genes involved in cell proliferation, survival and migration/invasion [9, 62, 191-195].

How LPA receptors link to transcriptional activation has become an ideal system to investigate the molecular mechanisms of LPA signal transduction. Many biological effects of GPCR have been thought to occur through transactivation of receptor tyrosine kinases, especially EGFR [181, 182]. In our previous studies, however, the effect of LPA on gene expression was much more potent than that of EGF itself. LPA indeed weakly transactivates EGFR as reflected by induction of low levels of phosphorylation of EGFR (Fig. 3.5 E of Chapter 3) which was in no means comparable to that stimulated by EGF. Intriguingly, the effects of LPA on gene expression were sensitive to inhibition of EGF, suggesting requirement of a permissive or parallel input from RTK in transducing LPA GPCR signals. In further support of this mode of crosstalk between GPCR and RTK, the dependence on EGFR could be overcome by co-stimulation of c-Met with HGF to provide an alternate RTK activity. In the current study, we explored the role of EGFR in LPA-induced activation of the transcription factors AP-1 and NF-κB. Our results indicate that activation of AP-1 components by LPA was highly sensitive to inhibition of EGFR while LPA stimulated NF-κB via an EGFR-independent manner, suggesting that EGFR activity is required for selective signaling cascades rather than proximal activation of LPA receptors. We further identified EGFR-dependent and independent G protein signaling cascades involved in activation of these transcription factors. Consistent with involvement of AP-1 in a multitude of biological processes, RTK activity is needed for LPA-induced cytokine production, cell proliferation, migration and invasion.

4.2 Materials and Methods

Materials Sources of materials were as described in previous chapters. 1-Oleoly (18:1) LPA was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Prior to use, these phospholipids were dissolved in PBS containing 0.5% fatty acid-free bovine serum albumin (BSA). BSA, Fugene 6 and protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). Plasmid DNA was purified using the endo-free purification kit from Qiagen (Valencia, CA). Luciferase assay reagents were obtained from Promega (Madison, WI). All oligonucleotides and primers were synthesized by Operon Biotechnologies, Inc (Huntsville, AL). QuikChange XL site directed mutageneis kit were purchased from Stratagene (La Jolla, CA). Anti-phospho p65, anti-phospho PKD and anti-tubulin α/β antibodies were obtained from Cell Signaling (Danvers, MA). Cell culture medium and lipofectamine 2000 were obtained from Invitrogen Inc. (Carlsbad, CA). Bovine fetal serum was from Biomeda (Foster City, CA). Enzymes were from New England biolab (Ipswich, MA) Hepatocyte growth factor (HGF) was obtained from R & D systems (Minneapolis, MN). Epidermal growth factor (EGF), AG1478, U73122, GF-109203X and anti-β-actin monoclonal antibody were obtained from Sigma-Aldrich (St. Louis, MO). All other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture The sources of ovarian cancer cell lines used in the study were described previously [9, 192]. These cells were cultured in RPMI medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. All cell lines were frozen at early passages and used for less than 10 weeks in continuous culture.

Nuclear Extract Preparation– LPA-stimulated or control cells were washing twice with cold PBS, harvested by scraping with a rubber policeman and centrifuged at 1000 rpm for 3 min. Cell pellets were resuspended in a hypotonic lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40], incubated for 15 min on ice, and centrifuged at 3000 rpm. The nuclei pellet was washed once with the hypotonic lysis buffer, resuspended in hypertonic nuclear lysis buffer (50 mM Tris-HCl (pH 8.3), 0.4M NaCl, 40% glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA) and further incubated for 10 mins before centrifugation at 13,000 rpm. The supernatant was collected and quick-frozen in liquid nitrogen before storage at -80°C. Protein concentration was determined with Pierce BCA kit.

Western Blot Cells were lysed in SDS sample buffer or in ice-cold X-100 lysis buffer [1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 100 mM NaF, 10 mM Na PPi, and protease inhibitor cocktail]. Total cellular proteins were resolved by SDSPAGE, transferred to Immun-Blot membrane [poly(vinylidene difluoride)] (BIO-RAD, Hercules, CA), and immunoblotted with antibodies following the protocols of manufacturers. Immunocomplexes were visualized

with an enhanced chemiluminescence detection kit from Amersham (Piscataway, NJ) using the horseradish peroxidase–conjugated secondary antibodies (Cell Signaling, Danvers, MA).

Plasmids Constructs A truncated EGFR cDNA (amino acids residues 1-677) lacking the cytosolic domain was cloned into pcDNA3 vector (Invitrogen, Carlsbad, CA) using primers: EGFR-Fwd 5' CATAAGCTTGGAGCAGCGATGCGACCCTCC 3' and DN-EGFR-rev 5'CATCTCGAGGCGCTTCCGAACGATGTGG3'. The AP-1 responsive luciferase vector pGL2-3xAP1-Luc was made by cloning three repeats of AP-1 consensus binding sequence (TGATGACTCAG) in front of the minimum TK promoter and the luciferase gene as we described previously [9]. The NF-κB responsive luciferase vector pGL2-3xNF-kB-Luc was obtained from Stratagene (La Jolla, CA). The vector contains three repeats of the NF-κB consensus sequence (GGGGACTTTCC) cloned into the pGL2-basic vector in front of a minimum TK promoter. The G_q dominant-negative mutant vector pLZRS-IRES-Gq(G208A) was kindly provided by Dr. E. Roos [250].

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

from Promega (Madison, WI). The luciferase activity was normalized on the basis of the activity of cotransfected β-galactosidase reporter driven by the cytomegalovirus promoter (pCMVβ-gal). Retroviral stock from EGFR-DN stable cells was a kind gift from Dr. Paul Dent, VCU Medical Centre.

Electromobility shift assay (EMSA)- AP-1 and NF-κB consensus oligonucleotides AP-1 sense 5'GGCGCTTGATGACTCAGCCGGAA 3'; AP-1 antisense 5'GGTTCCGGCTGAGTCATCAAGCG 3'; NF-κB sense 5'ATGTTGAGGGGACTTTCCCAGGCGG 3' and NF-κB antisense 5' GCCTGGGAAAGTCCCCTCAACTGG 3' were synthesized by Operon Biotechnologies and annealed in 20 mM Tris (pH 7.4), 1 mM dithiothreitol, 50 mM NaCl and 10 mM $MgCl_2$. Oligonucleotides were labeled at 3' end with [α-^{32}P]dCTP using klenow enzyme. For the gel shift assay, binding reaction was performed by incubating 4 μg of nuclear protein in gel shift buffer (10 mM HEPES (pH 7.8), 10% glycerol, 1 mM EDTA, 25 mM $MgCl_2$, 50 mM KCl, 1 μg of poly(dI.dC), 3 μg BSA and protease inhibitors) in a final volume of 20 μL for 10 mins at 25°C. Specificity of binding with each ^{32}P-random labeled probe was determined with 50-fold excess of unlabeled oligonucleotides. In supershift experiments, nuclear extracts were incubated for 15 min at room temperature with 1 μg of anti-c-Jun, anti-p65 or normal mouse IgG (Santa Cruz Biotech) prior to gel shift reaction. Complexes were separated by electrophoresis on 5% non-denaturing polyacrylamide gel

(PAGE). Gels were dried under vacuum and subjected to autoradiography using a Phosphoimager.

Cell Growth Assay- The growth of Caov-3 and Skov-3 were assayed by seeding cells in 6-well dishes at 3 to 5 x 10^4 cells per well in complete growth medium (RPMI supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin). After 48-72 h of culture, culture medium was replaced with serum-free medium with further incubation for 18 h. Cells were stimulated with or without 1 μM LPA and/or 1 μM AG1478 in fresh serum-free medium. Cell counts were performed on triplicate trypsinized cultures before and post stimulation using a cell counter (Model ZI; Coulter Electronics, Hialeah, FL).

Migration Assay- The migration of Skov-3 cells was assayed using transwell chambers (pore size 8 μM) (BD Biosciences, Bedford, MA). The inserts were precoated with collagen. LPA (10 μM or 1 μM) or vehicle was added to the lower chamber. Serum-starved cells (1 x 10^5) were loaded to the upper chamber with or without AG1478 at a final concentration of 1 μM. Non-migrated cells were removed from the top filter surface with a cotton swab. Migrated cells attached to the underside of the transwells were washed with PBS, stained with crystal violet and counted under a microscope.

The invasion of SKOV-3 cells was measured using Transwells coated with growth factor–reduced Matrigel Basement Membrane Matrix (pore size 8 μM; BD Biosciences;

cat. no. 354483). The procedure for invasion was similar to that of the migration assay described above except that cells were allowed to invade for 20-24 h at 37°C.

Scratch (Wound Closure) Assay- Confluent monolayers of Caov-3 were serum starved for 18 hr. Scratches were made using sterile 1 μl pipette tips. Displaced cell debris was washed off with serum-free media before stimulation with 5 μM LPA or BSA (vehicle) with or without 1 μM AG1478. Images of wounded areas were captured at 0 h and 16 h after addition of LPA.

Rho and Ras Activation Assays- Activation of Rho and Ras were analyzed by glutathione S-transferase (GST) pulldown assays [251]. The cells were grown in 10-cm dishes to subconfluence, starved overnight, and stimulated with LPA or vehicle for the indicated periods of time. The cells were lysed in Magnesium-containing lysis buffer (MLB: 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP40, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Clarified lysates were incubated for 45-60 min at 4°C with GST-Rhotekin-RBD (Rho binding domain of Rhotekin, residues 7-89; [252]) or GST-Raf-RBD (Ras binding domain of Raf, residues 1-149[253]) produced in Escherichia coli and immobilized on glutathione-coupled Sepharose beads. Beads were washed in MLB three times, eluted with SDS sample buffer, and analyzed by Western blotting using monoclonal anti-Rac antibody (BD Biosciences; Cat. No. 610650) or rabbit anti-Ras antibody (Cell signaling, Denvers, MA).

Densitometry and Statistics- Intensities of western blot bands were quantified using the NIH Image J software. All numerical data were presented as mean ± SD. The statistical significance of differences was analyzed using Student's *t* test where $P < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 Activation AP-1 Proteins by LPA

LPA is a master inter-cellular regulator of gene expression in mammalian cells. Although post-transcriptional regulation may be involved in the reinforcement of the effect LPA on gene expression, the initial input is driven by transcriptional activation. We have previously shown that the effects of LPA on gene expression are mediated by a number of prominent transcription factors including AP-1, NF-κB, C/EBP and Sp-1 [9, 232, 254]. Thus activation of transcription factors offers an ideal readout to study functions of LPA receptors, their downstream signaling networks and their crosstalk with RTKs. In ovarian and other cancer cell lines expressing LPA receptors, treatment with LPA led to induction of various AP-1 proteins.

As demonstrated in Fig. 4.1 A, LPA induced c-Jun, Fos, Jun B and Fra-1 expression in a time-dependent manner in Caov-3 cells. Induction of c-Jun and Fos expression occurred immediately and peaked at 1 hr after exposure to LPA. Jun B and Fra-1 were induced at later hours and highest levels were seen at 4-6 hr of LPA treatment (Fig. 4.1). The sequential induction of these AP-1 components could lead to sustained increases

in AP-1 activity. Indeed, EMSA confirmed elevation in AP-1 DNA-binding activity in LPA-treated cells which lasted for many hours (Fig. 4.1 B). Consistent with delayed induction of Jun B and Fra-1, the peak DNA-binding activity was detected after 4-6 hr of treatment with LPA. We further confirmed that LPA treatment resulted in transcriptional activation. Caov-3 cells were transfected with the AP-1 responsive luciferase reporter pGL2-3xAP-1-Luc. Treatment with LPA induced more than 25 fold increases in luciferase activity compared to untreated cells (Fig. 4.1 C).

In these experiments, we also analyzed EGF and HGF for their ability to activate AP-1. The effects of LPA on AP-1 protein expression and AP-1 DNA-binding activity were stronger or at least comparable to those of EGF (Fig. 4.2 A and C). Since LPA induced only minimal activation of EGFR as reflected by weak phosphorylation at Y-1068 (Fig. 3.5 E of Chapter 3), it is unlikely that LPA stimulated AP-1 through transactivation of EGFR. Compared to HGF, LPA was much more efficacious inducer of each of AP-1 proteins and AP-1 DNA-binding activity (Fig. 4.2 B and C). For example, LPA triggered expression of Fos and Fra-1 while HGF failed to stimulate their expression.

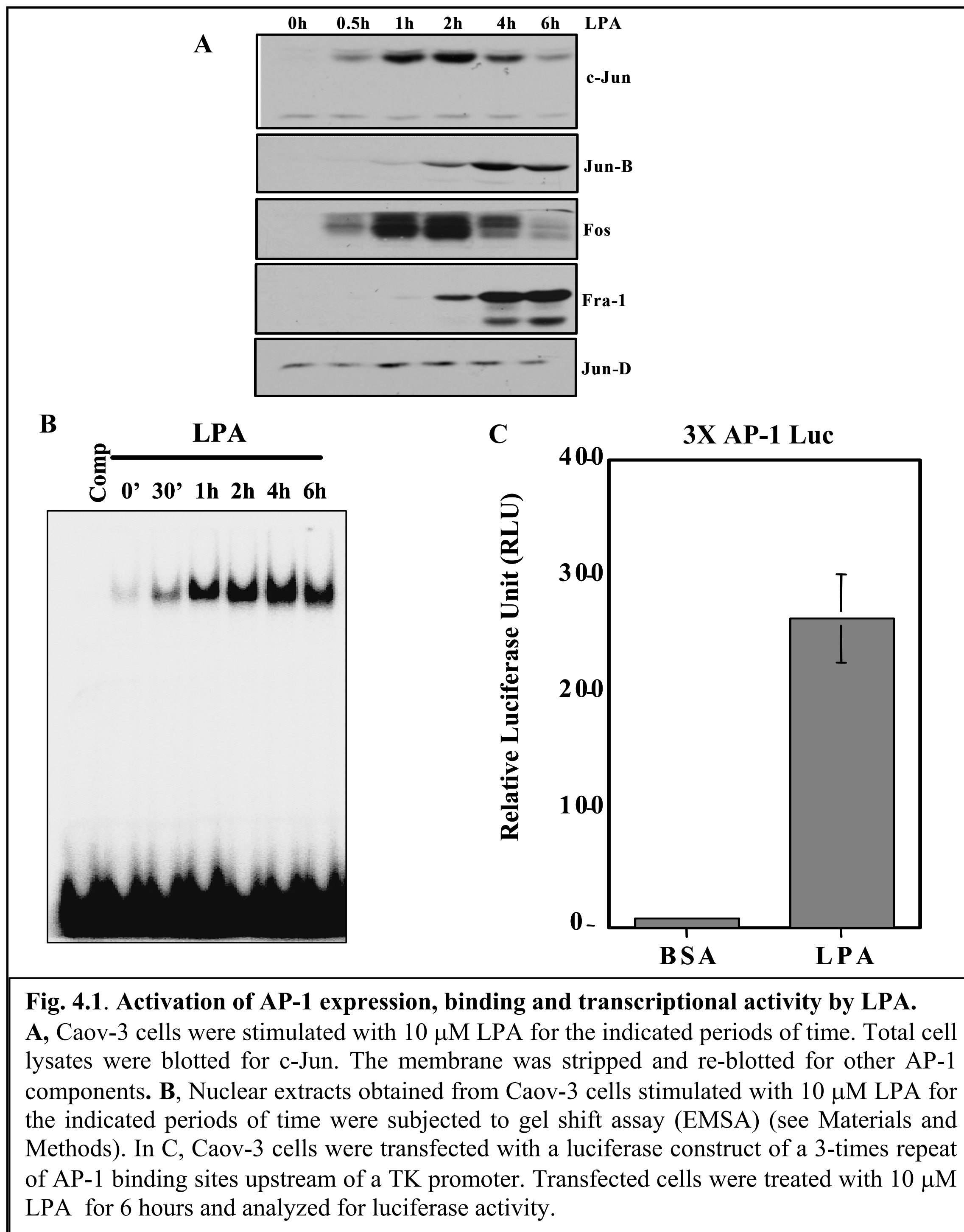


Fig. 4.1. **Activation of AP-1 expression, binding and transcriptional activity by LPA.**
A, Caov-3 cells were stimulated with 10 μM LPA for the indicated periods of time. Total cell lysates were blotted for c-Jun. The membrane was stripped and re-blotted for other AP-1 components**. B**, Nuclear extracts obtained from Caov-3 cells stimulated with 10 μM LPA for the indicated periods of time were subjected to gel shift assay (EMSA) (see Materials and Methods). In C, Caov-3 cells were transfected with a luciferase construct of a 3-times repeat of AP-1 binding sites upstream of a TK promoter. Transfected cells were treated with 10 μM LPA for 6 hours and analyzed for luciferase activity.

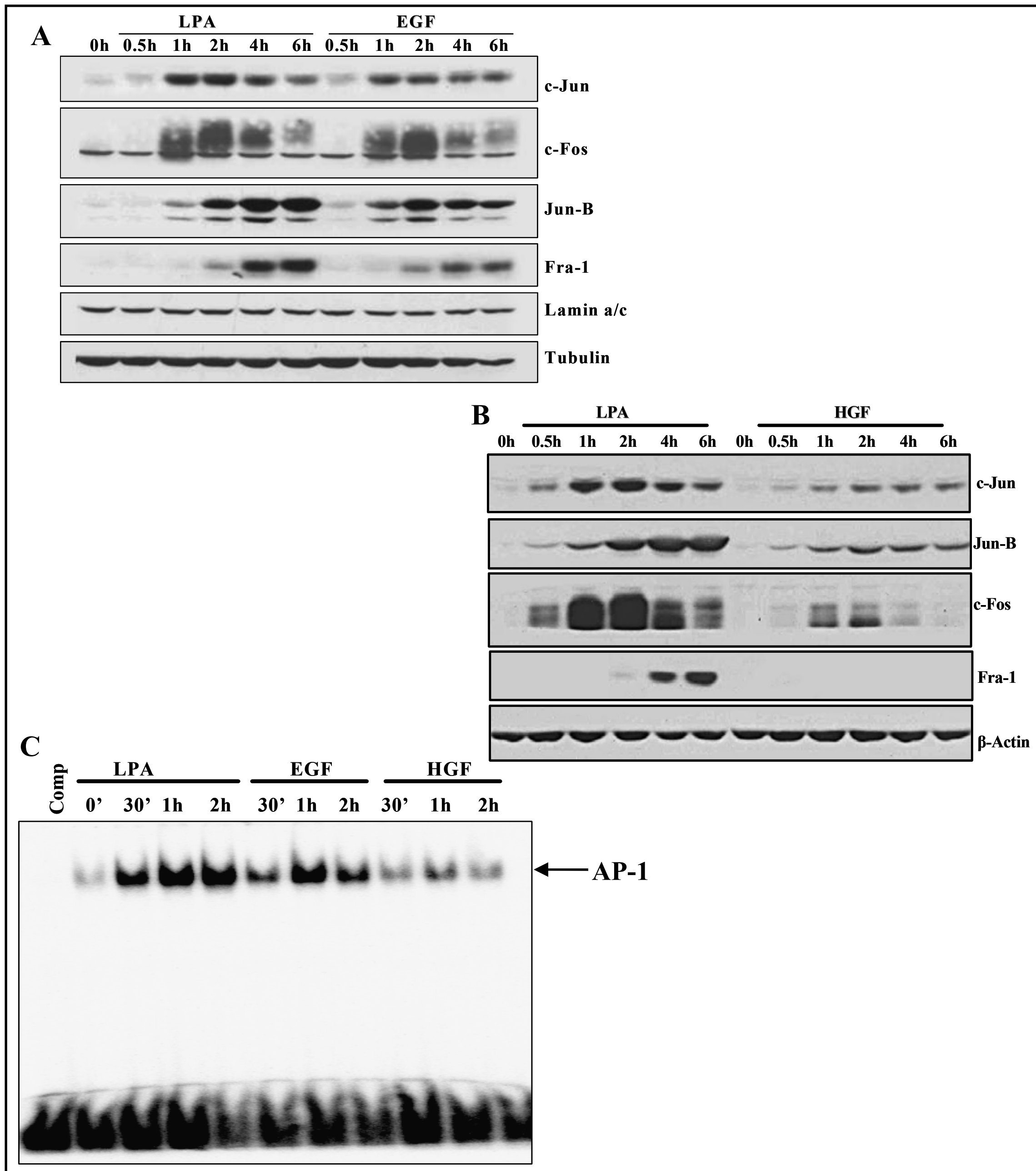


Fig. 4.2. **Comparison of LPA to RTK agonists in activation of AP-1.**
Caov-3 cells were stimulated with 10 μM LPA, 25 ng/ml EGF or 25 ng/ml HGF for the indicated periods of time and analyzed by western blotting for expression of AP-1 proteins (**A** and **B**). Nuclear extracts obtained from Caov-3 cells stimulated with LPA, EGF or HGF for the indicated periods of time were subjected to EMSA using ^{32}P-labeled AP-1 consensus oligonucleotides (**C**).

4.3.2 Requirement of EGFR or an alternate RTK for LPA-induced activation of AP-1

Having observed the dependence of LPA-induced C/EBP-β activation on RTK activity, we asked if this obligatory role was a general requirement for activation of transcription factors by LPA. Pretreatment of ovarian cancer cells with AG1478, a specific pharmacological inhibitor of EGFR kinase activity, abrogated LPA-induced expression of JunB and Fra-1 (Fig. 4.3 A). The expression of c-Jun and c-Fos was dramatically inhibited by the presence of AG1478. Similarly, LPA-induced AP-1 DNA binding and transcriptional activities were drastically suppressed by AG1478 (Fig. 4.3 B and C)

To determine whether EGFR is specifically needed for GPCR signaling to AP-1, we co-stimulated Caov-3 cells with LPA and HGF in the presence of AG1478. The stimulatory effects of LPA on expression of AP-1 proteins and AP-1 DNA-binding activity were fully recovered by co-treatment of the cells with HGF to activate the c-Met, another receptor tyrosine kinase (Fig. 4.3 A and B). The role of HGF was not due to activation of AP-1 by HGF itself as the effects of HGF on AP-1 protein expression and DNA-binding activity were marginal compared to those of LPA (Fig. 4.2 B and C). The restoration of AP-1 activity by HGF also demonstrated that AG1478 did not block LPA-induced AP-1 activation through nonspecific or toxic effects. These results indicate that activity of a RTK, not necessarily EGFR, provides an activity indispensable for transmission of GPCR signals to AP-1 although such an activity itself is insufficient for full activation of AP-1.

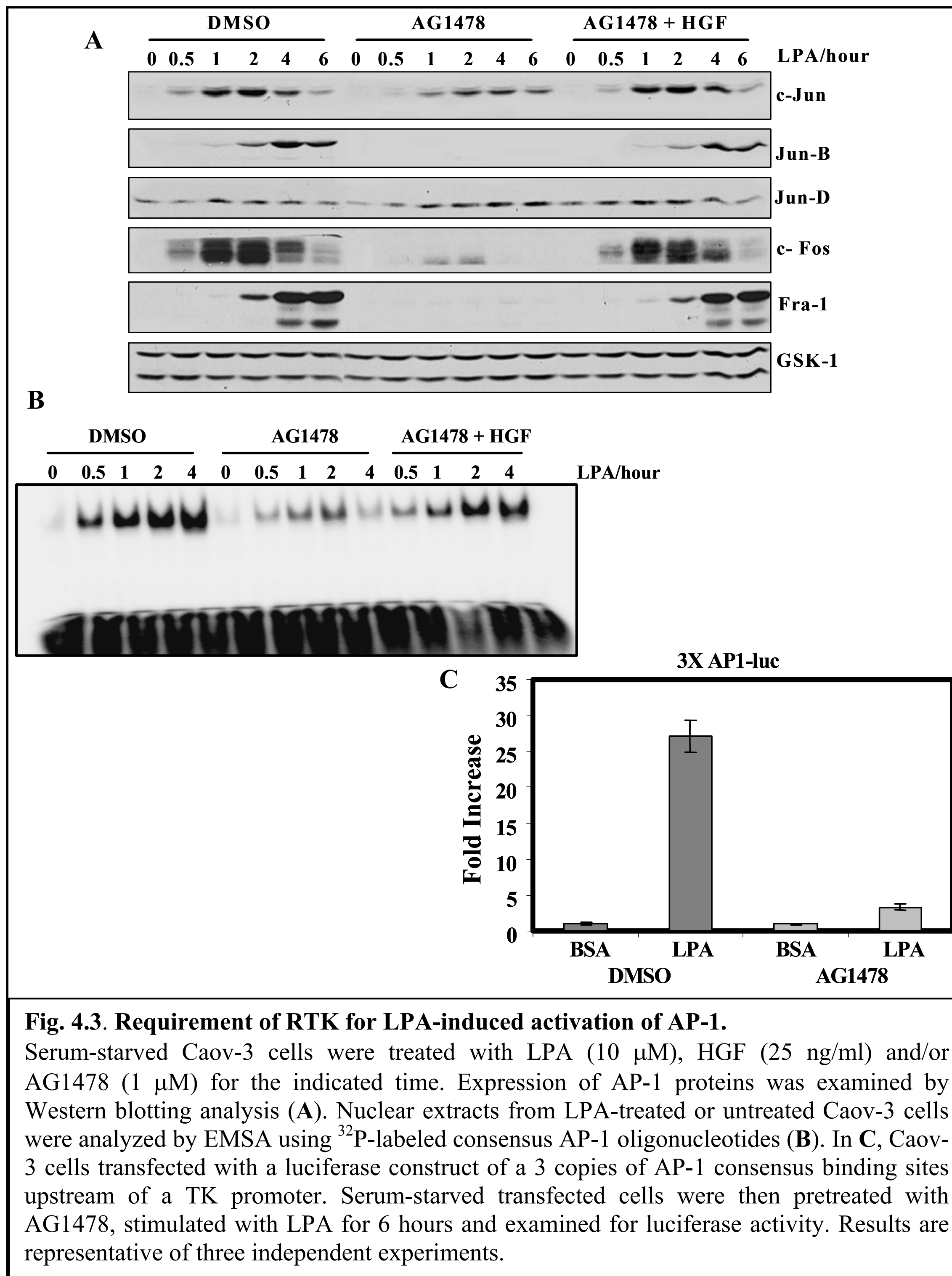


Fig. 4.3. **Requirement of RTK for LPA-induced activation of AP-1.**
Serum-starved Caov-3 cells were treated with LPA (10 μM), HGF (25 ng/ml) and/or AG1478 (1 μM) for the indicated time. Expression of AP-1 proteins was examined by Western blotting analysis (**A**). Nuclear extracts from LPA-treated or untreated Caov-3 cells were analyzed by EMSA using ^{32}P-labeled consensus AP-1 oligonucleotides (**B**). In **C**, Caov-3 cells transfected with a luciferase construct of a 3 copies of AP-1 consensus binding sites upstream of a TK promoter. Serum-starved transfected cells were then pretreated with AG1478, stimulated with LPA for 6 hours and examined for luciferase activity. Results are representative of three independent experiments.

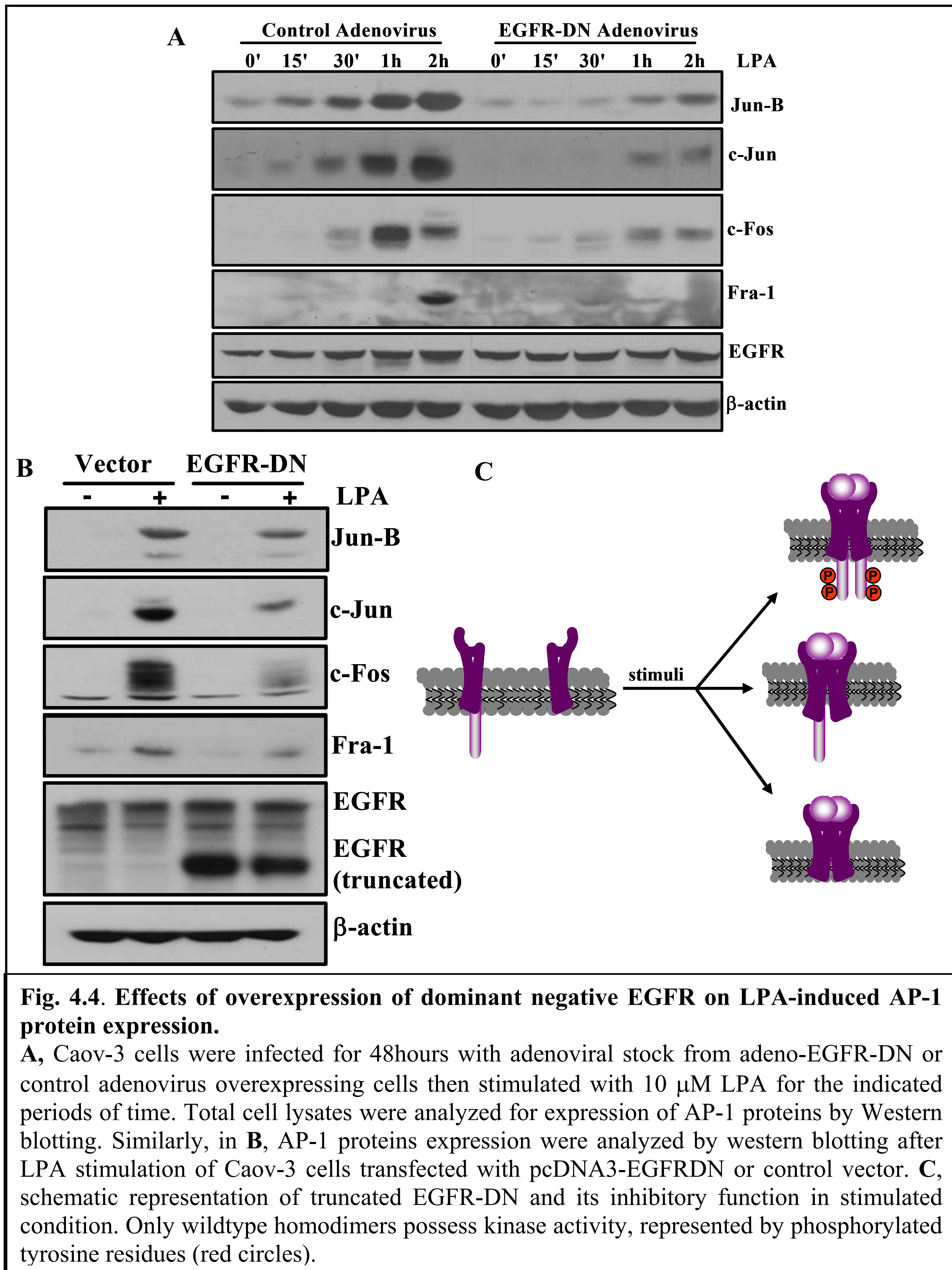


Fig. 4.4. Effects of overexpression of dominant negative EGFR on LPA-induced AP-1 protein expression.
A, Caov-3 cells were infected for 48hours with adenoviral stock from adeno-EGFR-DN or control adenovirus overexpressing cells then stimulated with 10 µM LPA for the indicated periods of time. Total cell lysates were analyzed for expression of AP-1 proteins by Western blotting. Similarly, in **B**, AP-1 proteins expression were analyzed by western blotting after LPA stimulation of Caov-3 cells transfected with pcDNA3-EGFRDN or control vector. **C**, schematic representation of truncated EGFR-DN and its inhibitory function in stimulated condition. Only wildtype homodimers possess kinase activity, represented by phosphorylated tyrosine residues (red circles).

In further support of these results derived from pharmacological inhibition of EGFR, overexpression of a dominant negative, truncated form of EGFR (EGFR-DN) [255] in Caov-3 cells by both adenoviral transduction and direct expression plasmid suppressed LPA-induced expression of each of AP-1 proteins (Fig. 4.4). These pharmacological and molecular approaches together establish an obligatory role for EGFR or an alternate RTK in activation of the AP-1 transcription factor by LPA.

4.3.3 EGFR-independent activation of NF-κB by LPA

The role of EGFR in LPA activation of CEBP-β (Chapter 3) and AP-1 raises the possibility that a basal RTK activity might be ubiquitously required for GPCR actions. This could be due to the requirement of RTK activity for activation of GPCR itself. If the RTK input is involved in activation of the specific intracellular G protein signaling processes instead of GPCR activation on the membrane, some LPA signaling pathways may not rely on RTK activity. To distinguish these possibilities, we examined LPA-induced activation of another transcription factor, NF-κB, a transcription factor critical for activation of many LPA target genes involved in inflammation and cancer progression. As shown in Fig. 4.5 A-C, in Caov-3 and Skov-3 cells treated with AG1478 to block EGFR function, LPA induced NF-κB p65 phosphorylation, IκB phosphorylation and IκB degradation at levels comparable to those in control cells untreated with AG1478.

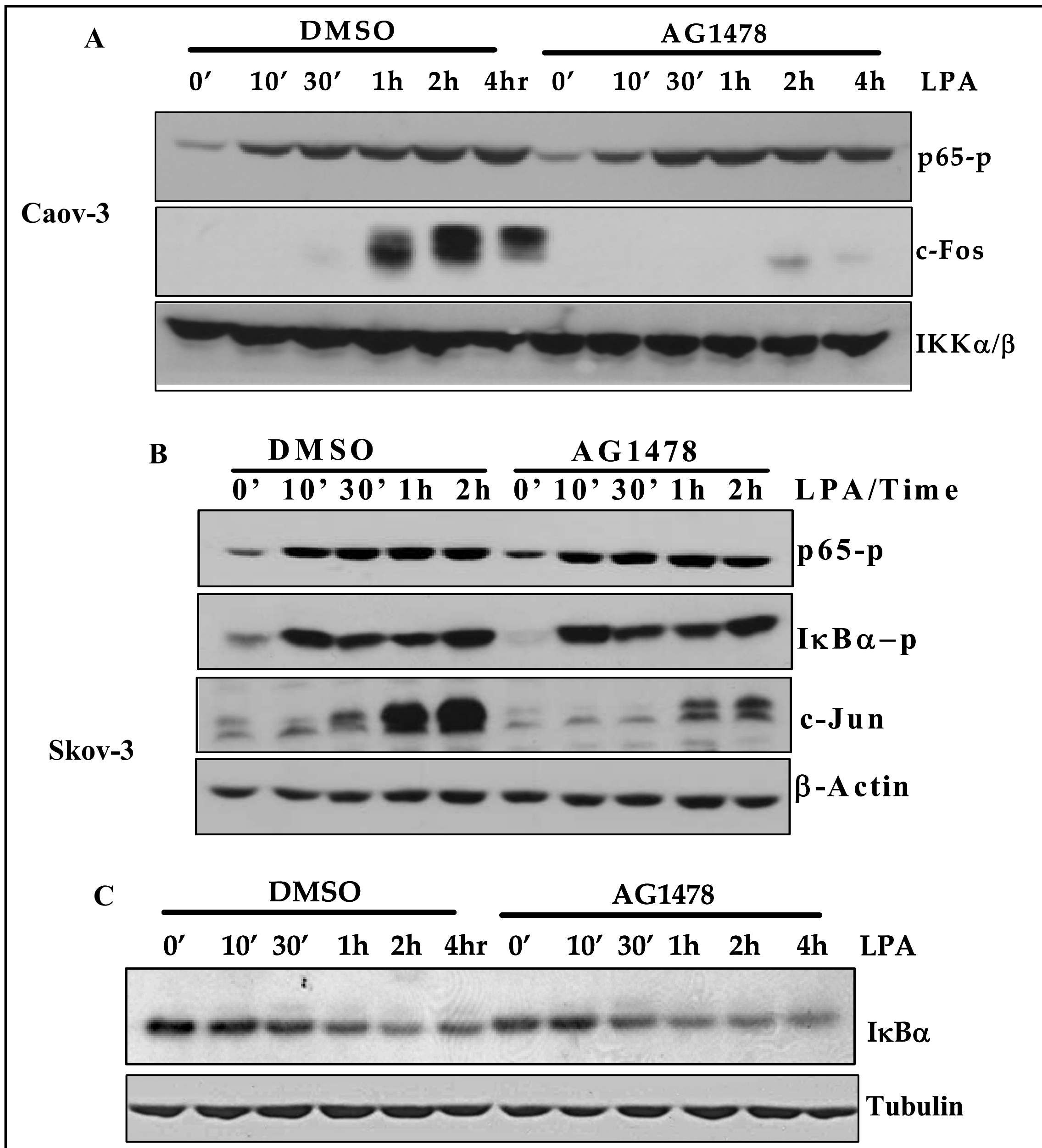


Fig. 4.5. **EGFR-independent activation of NF-κB by LPA.**
Total cell lysates of serum-starved Caov-3 (**A and C**) or Skov-3 cells (**B**) stimulated with 10 μM LPA and/or 1 μM AG1478 for the indicated periods of time were analyzed by Western blotting for phosphorylated NF-κB p65. The membrane was reprobed with anti-phospho-IκBα (**B**) or anti-IκBα (**C**). IKKα/β, β-actin or Tubulin were used to show equal loading. c-Jun or c-Fos expression was used to confirm efficacy of batch of AG1478 used in experiment.

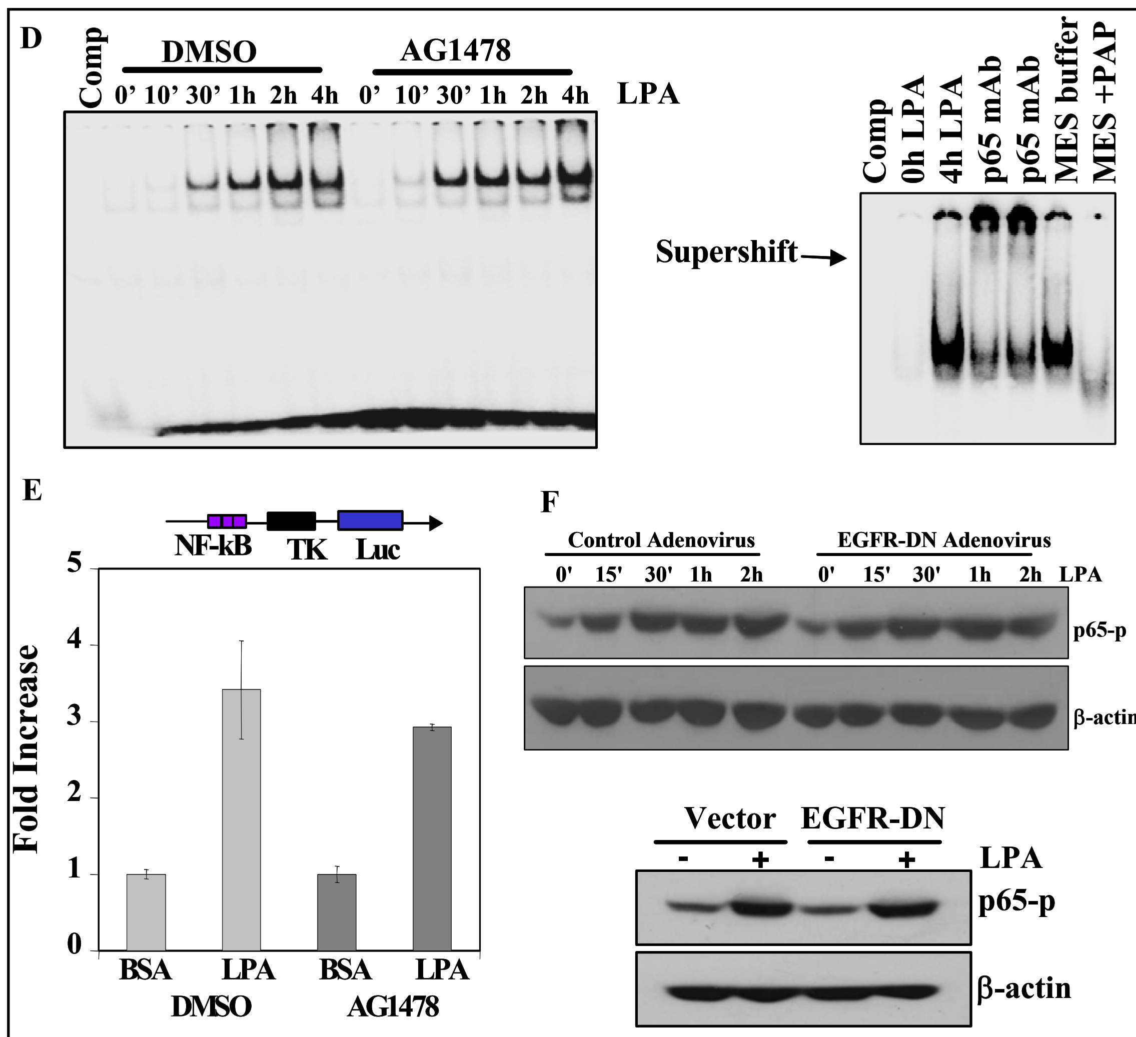


Fig. 4.5 (cont'd). **EGFR-independent activation of NF-kB by LPA**

D, Nuclear extracts obtained from Caov-3 cells stimulated with 10 μM LPA or 1 μM AG1478 for the indicated periods of time were subjected to EMSA. Supershift experiments were performed on 4-h LPA-treated nuclear extracts using 2 and 5 μg anti-p65 mouse monoclonal antibody. For dephosphorylation of nuclear extracts, 1 unit of potato alkaline phosphatase (PAP) was added for 15 min prior to binding reaction In **E**, Caov-3 cells were transfected with a luciferase construct of a 3-times repeat of NF-κB binding sites upstream of a TK promoter. Transfected cells were treated with 10 μM LPA for 6 hours and analyzed for luciferase activity. **F**, Caov-3 cells were infected for 48 hours with adenoviral stock from adeno-EGFR-DN or control adenovirus overexpressing cells at 30 MOI, then stimulated with 10 μM LPA for the indicated periods of time (*upper*). In similar experiments, Caov-3 cells were transfected with pcDNA3-EGFR-DN or control vector (*lower*) and stimulated with LPA for 2 hrs. Total cell lysates were analyzed for phosphorylated p65 by Western blotting.

Similarly, LPA-stimulated NF-κB DNA-binding activity was not compromised by AG1478 as measured by EMSA (Fig. 4.5 D); nor was LPA-driven NF-κB transcriptional activity significantly ablated by incubation of cells with AG1478 as analyzed by the NF-κB responsive luciferase reporter assay (Fig. 4.5 E). Further, overexpression of dominant negative EGFR (EGFR-DN) did not interfere with LPA-induced p65 phosphorylation (Fig. 4.5 F) despite strong inhibitory effect of EGFR-DN on AP-1 protein expression (Fig. 4.4). Therefore, in sharp contrast to AP-1 activation, LPA-elicited NF-κB activity occurs via an EGFR-independent route. The results also indicate that the crosstalk with RTK is required only for a selective subset of biochemical events but not overall activities of LPA receptors.

4.3.4 G protein cascades mediating LPA-induced AP-1 and NF-κB activation

To identify the mechanism for the differential requirements of RTK in transmitting GPCR signals to AP-1 and NF-κB, we examined G protein signaling cascades responsible for activating AP-1 and NF-κB. The classical Edg LPA receptors expressed in cancer cell lines couple to G_i, G_q and $G_{12/13}$ [36-42]. Inhibition of G_i with pertussis toxin (PTX) strongly decreased LPA-induced AP-1 proteins c-Jun and c-Fos as shown in Fig. 4.6 A, indicating that G_i signaling links to AP-1 activation by LPA. However, G_i was disposable for NF-κB activation as PTX did not interfere with NF-κB p65 phosphorylation induced by LPA.

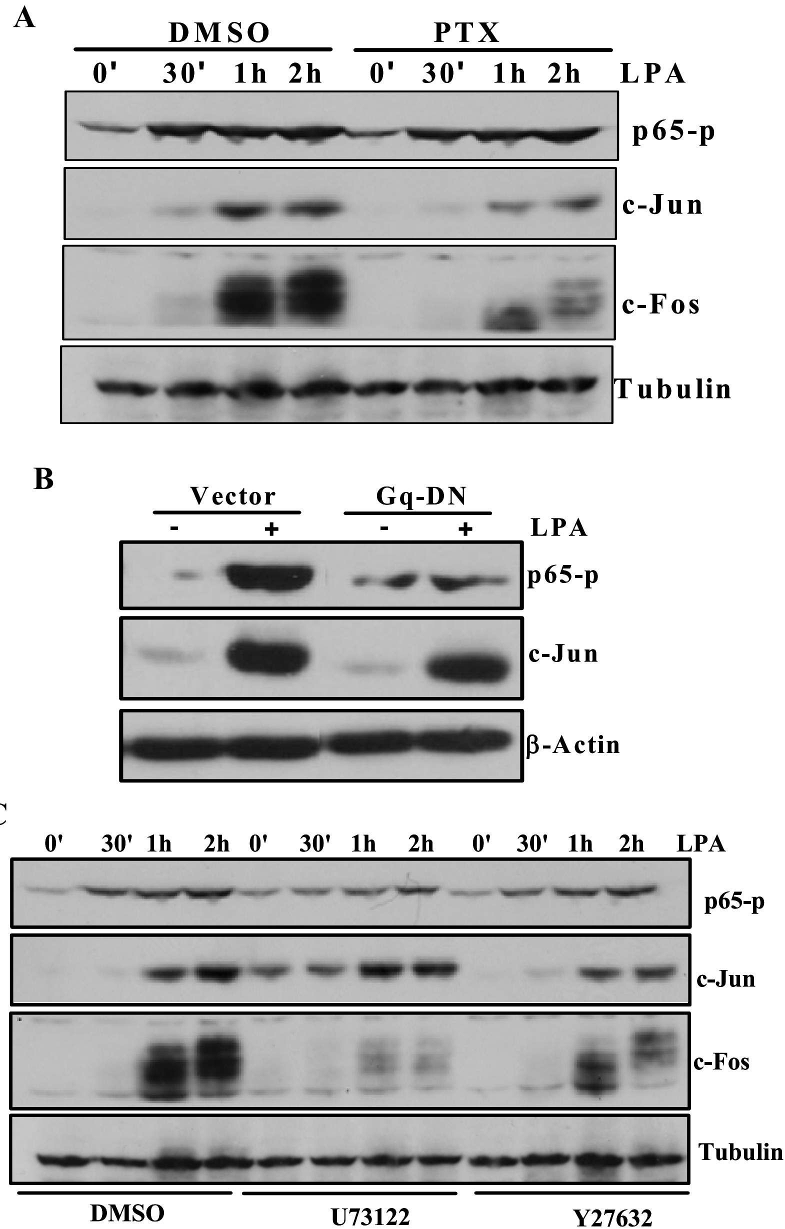


Fig. 4.6. **G proteins cascades mediating LPA-induced AP-1 and NF-κB activation**
Caov-3 cells were stimulated with 10 μM LPA and/or, in **A,** 25 ng/ml PTX (G_i inhibitor), 5 μM U73122 (PLCγ inhibitor) or 10 μM Y27632 (ROCK inhibitor) (**C**) for the indicated periods of time. Total cell lysates were analyzed by Western blotting for phospho-p65, c-Jun or c-Fos. In **B**, Caov-3 cells were transfected with expression plasmid, pcDNA3-GqG208A, dominant negative of G_q. Transfected cells were treated with 10 μM LPA for 6 hours and analyzed by Western blotting.

To assess the contributions of G_q signaling cascade to AP-1 and NF-κB activation, we took advantage of a dominant negative form of G_q (G208A) that has been shown to specifically block G_q–mediated pathways in different cell systems [250, 256, 257]. The GqG208A was transfected into Caov-3 cells using Amaxa nuocleofector Kit T that yield high transfection efficiency in ovarian cancer cell lines as we described previously (Chapter 2, Materials and Methods). Expression of the GqG208A mutant almost completely prevented LPA-induced p65 phosphorylation but not c-Jun expression (Fig. 4.6 B). Induction of Fos by LPA was decreased by overeexpression of GqG208A. Due to the lack of commercially available G_q pharmacological inhibitors, we made use of U73122, an antagonist of PLC that lies downstream of G_q [16, 49]. U73122 inhibited LPA-induced NF-κB p65 phosphorylation and c-Fos induction (Fig. 4.6 C). Only slight reduction in LPA-induced c-Jun expression was observed in U73122-treated cells. These data establish that the G_q-mediated signaling is critical for NF-κB activation in response to LPA. Activation of G_q may also contribute to LPA induction of certain AP-1 proteins such as c-Fos.

We also examined the role of $G_{12/13}$ in LPA-mediated activation of AP-1 and NF-κB through inhibition of the $G_{12/13}$ effector ROCK. ROCK has been reported to participate in LPA-induced c-Jun expression in NIH 3T3 cells [258]. We examined the effects of a highly selective ROCK inhibitor Y-27632 on LPA-induced AP-1 and NF-κB activation. The compound did not affect LPA-induced p65 phosphorylation but compromised c-Jun and c-Fos induction (Fig. 4.6 C). Based on these results, each of G protein modules (G_i,

G_q, and $G_{12/13}$) seems to contribute to AP-1 activity while only the G_q pathway couples to NF-κB activation in LPA-stimulated cells.

4.3.5 Differential requirement of EGFR for activation of G signaling cascades

We next explored whether EGFR is differentially required for activation of intracellular G protein signaling modules. Since it is practically difficult to analyze activation of G proteins, we examined the downstream effectors of each class of G proteins coupled to LPA receptors. Ras activation is a well-defined G_i-dependent signal, Rho lies downstream of $G_{12/13}$ and activation of G_q could be monitored by analyzing the PKC-PKD pathway. As demonstrated in Fig. 4.7 A, LPA-induced Ras activation was completely blocked by AG1478 as measured by GST pulldown assay for the GTP-bound Ras (GTP-Ras). In agreement with EGFR-dependent activation of Ras by LPA, Erk activation was also highly sensitive to AG1478 (Fig. 4.7 B), suggesting that LPA-induced activation of G_i relies on a permissive signal from EGFR. However, the GST pulldown analysis of Rho activation demonstrated that EGFR was not involved in activation of $G_{12/13}$ as the downstream Rho was fully activated by LPA in the presence of AG1478 (Fig. 4.7 C). PKD is a well characterized substrate of various isoforms of PKC [259]. LPA stimulated a rapid and sustained PKD phosphorylation at Serine 916 (Fig. 4.8A). Blockade of PKC with GF-109203X specific inhibitor of classical PKCs, or inhibition of PLCγ with U73122 (Fig. 4.8 C) prevented LPA-induced phosphorylation of PKD, confirming that the PLC-PKC cascade lies upstream of PKD.

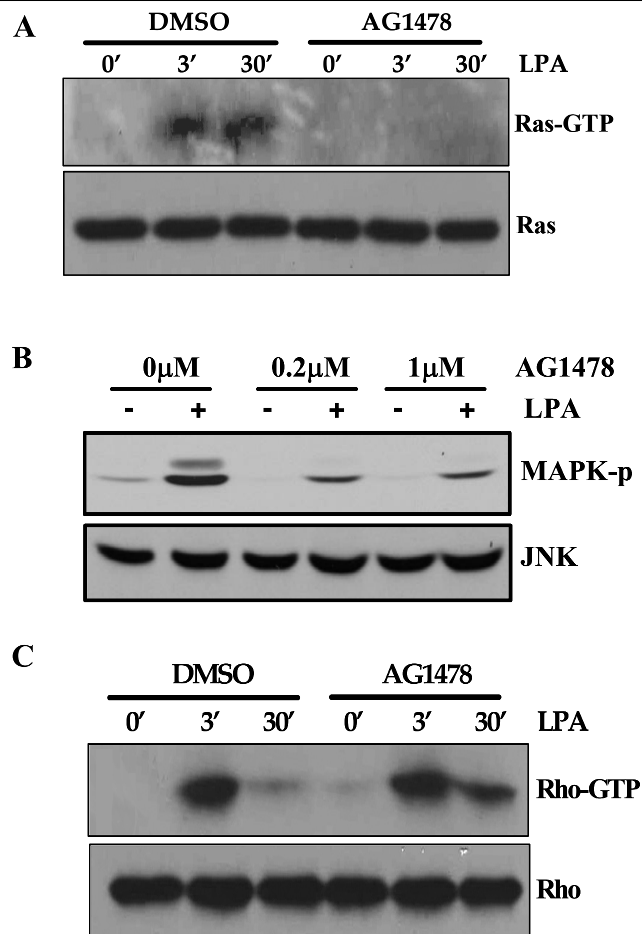


Fig. 4.7 Differential effects of EGFR inhibition on Ras and Rho activation by LPA
Serum-starved Caov-3 cells were stimulated with 10 μM LPA and/or 1 μM AG1478 for the indicated periods of time. Ras (**A**) or Rho (**C**) in cell lysates was concentrated by glutathione S-transferase (GST) pulldown assays (see Materials and Methods). Samples from pull-down assay and inputs were analyzed by Western blotting. In **B,** Caov-3 cells treated with LPA and/or indicated dose of AG1478 for 4 hours were analyzed for phosphorylated MAPK (anti-phospho Erk) by Western blotting.

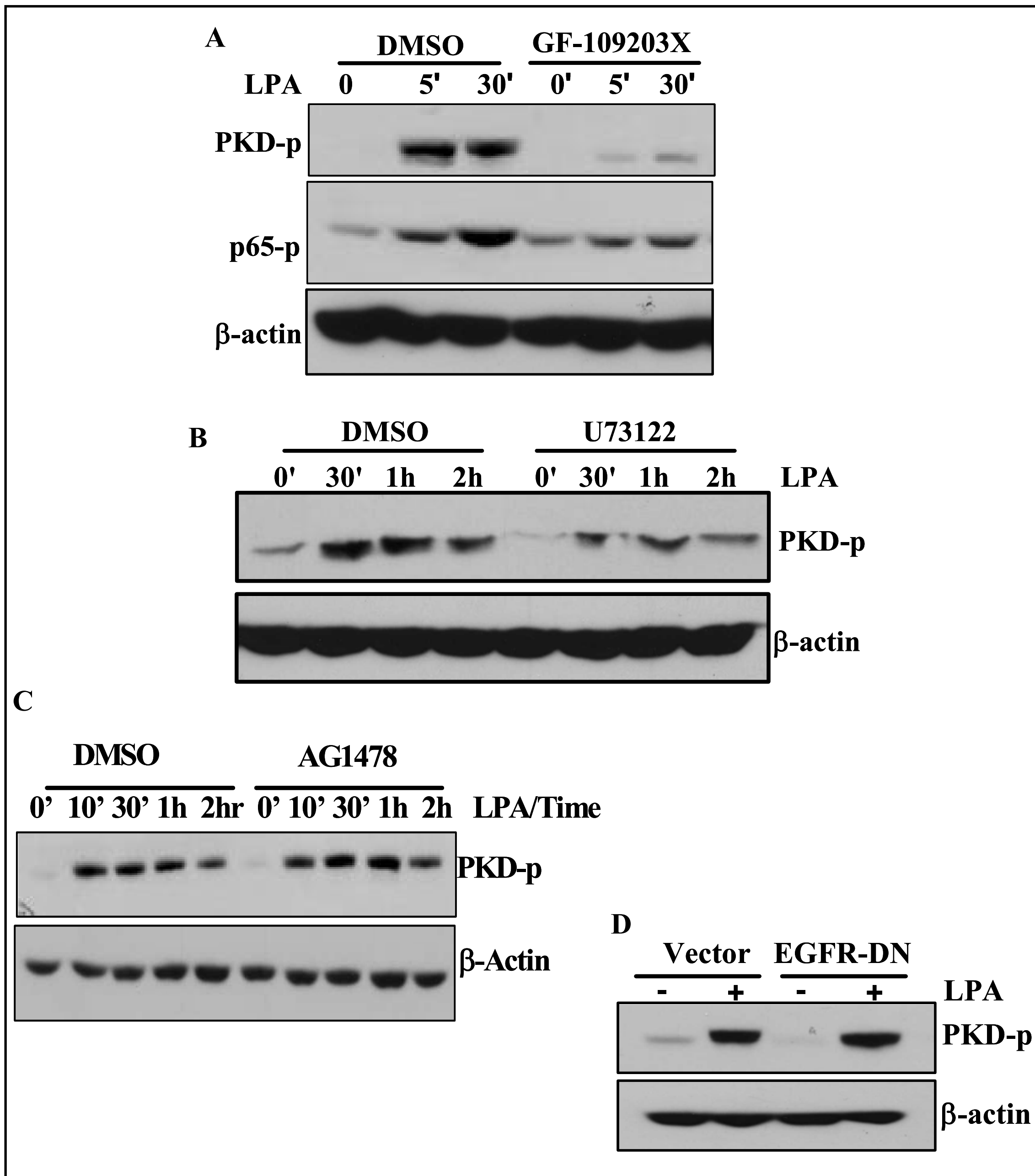


Fig. 4.8. **EGFR-independent activation of G_q signaling pathway by LPA.**
Serum-starved Caov-3 cells were stimulated with 10 μM LPA and/or 0.2 μM GF109203X, 10 μM U73122 or 1 μM AG1478 for the indicated periods of time. Total cell lysate were analyzed by Western blotting with anti-phosphoPKD (Ser 916) (**A-C**) In **D**, Caov-3 cells were transfected with pcDNA3-EGFRDN or control vector, stimulated with 10 μM LPA for 2hrs and subjected to Western blotting analysis. β-actin was used as control for equal

However, inhibition of EGFR function with AG1478 or EGFR-DN did not affect PKD phosphorylation induced by LPA (Fig. 4.8 C and D). Therefore, activation of the G_q-PLC-PKC-PKD pathway does not require EGFR activity. These results reveal EGFR-dependent G_i and EGFR-independent G_q and $G_{12/13}$ signaling cascades downstream of LPA receptors. Since these G protein pathways are linked to activation of specific transcription factors as specified above, these results provide a molecular basis for the differential requirements of EGFR in LPA-stimulated activation of the AP-1 and NF-κB transcription factors.

4.3.6 Essential roles of EGFR in multiple biological responses to LPA

If G_i and the downstream AP-1 depend on EGFR for activation, we expected that many cellular processes mediated by G_i or AP-1 are sensitive to EGFR inhibition. To further test this speculation, we first examined the effect of EGFR inhibition on LPA-induced IL-8 production, a functional outcome of synergistic actions of NF-κB and AP-1 as we described previously [9]. As demonstrated in Fig. 4.9 A, the prominent effect of LPA on IL-8 production was suppressed by inhibition of EGFR with AG1478, consistent with the necessity of EGFR for LPA-induced AP-1 activation. Further analysis of other cellular responses to LPA demonstrated that inhibition of EGFR suppressed LPA-afforded cell growth (Fig. 4.9 B). LPA-mediated migration, invasion and wound closure in ovarian cancer cell lines were also attenuated in the presence of AG1478 (Fig. 4.9 C-E), in support

of essential roles of EGFR-dependent G_i and its downstream signaling pathways in promotion of a broad range of cellular responses to LPA [18, 260].

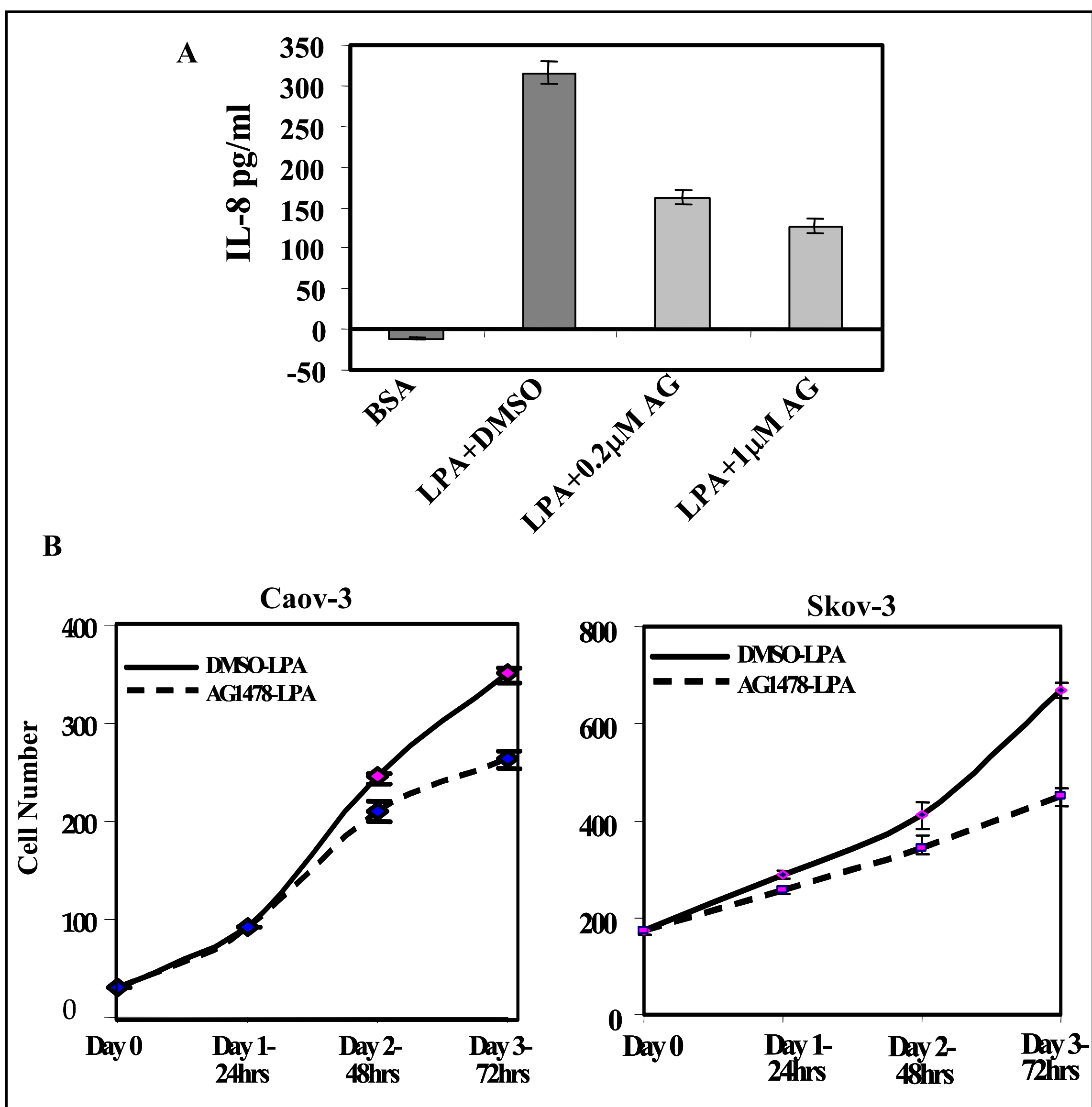


Fig. 4. 9. **Role of EGFR in LPA-mediated biological responses.**
A, Cultured supernatants of serum-starved Caov-3 cells pretreated with or without 1 µM AG1478 and stimulated with 10 µM LPA for 18hrs were assayed for IL-6 concentrations by ELISA. In **B**, triplicate samples of Caov-3 or Skov-3 cells were serum starved for 12hrs, stimulated with 5 µM LPA and/or 1 µM AG1478 for the indicated period of time. Cells were trypsinized and quantified in a cell counter.

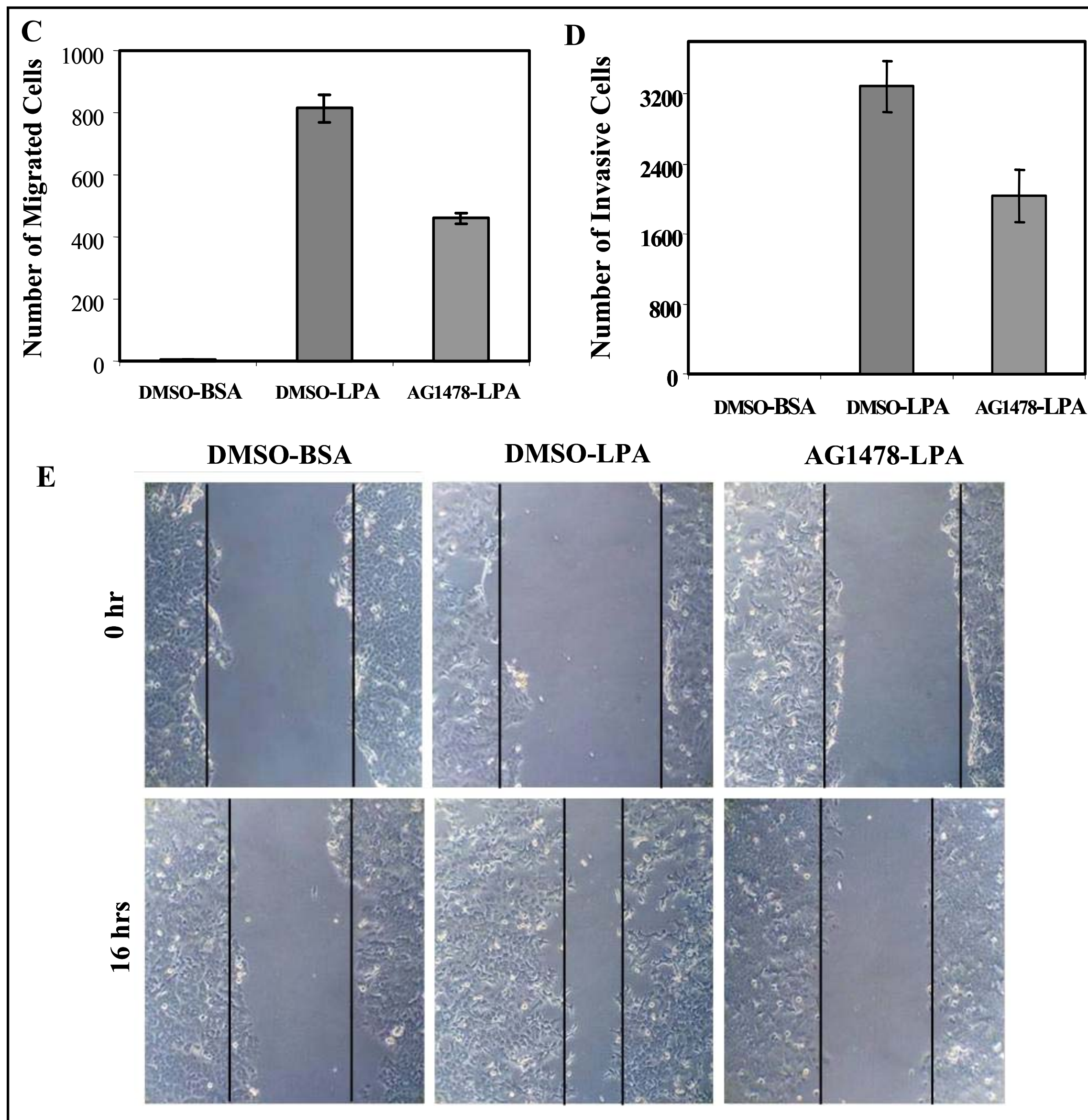


Fig. 4.9 (cont'd). **Role of EGFR in LPA-mediated biological responses**
Caov-3 cells were serum starved and seeded in collagen-coated (**C**) or growth factor–reduced matrigel basement membrane matrix (**D**) transwell chambers (see Materials and Methods). Migration or invasion of cells were assessed by stimulating cells with 10 μM LPA and/or 1 μM AG1478 for 6 and 24 hours respectively. Number of migrated or invaded cells was counted in 8 randomly selected fields across membrane. **E**, serum-starved confluent Caov-3 cells were scratched with sterile pipette and stimulated with LPA and/or AG1478. Images were captured immediately after scratch and 16 hours post-stimulation. Data shown are representative of three independent experiments.

4.4 Discussion

We have previously shown that LPA induces expression of multiple cancer-associated genes via activation of transcription factors [9, 232, 254]. In Chapter 2 and 3, we used LPA-induced Cox-2 expression as a model system to identify transcription factors and signaling networks involved in activation of Cox-2 expression by LPA. Through these studies, we demonstrated that CEBP-β is a key transcription factor responsible for initiating transcription of Cox-2. The activation of CEBP-β by LPA results from integration of signals from LPA receptor(s) and a permissive activity from RTK. To substantiate this crosstalk between GPCR and EGFR in transmission of GPCR signals, we have devoted the study in this Chapter to determining whether the EGFR signal is generally required for activation of other transcription factors by LPA. The results presented here indicate that LPA-induced activation of AP-1 relies on such an activity from EGFR in analogy to LPA-mediated C/EBP-β activation while LPA stimulates NF-κB in an EGFR-independent manner. The differential requirements of EGFR for AP-1 and NF-κB activation suggest that the EGFR signal is involved in activation of specific intracellular signaling cascades downstream of LPA receptors rather than proximal activation of LPA receptors on the membrane. Furthermore, we identified the intracellular G signaling cascades that interact with EGFR. Our results revealed that the G_i-mediated pathway relies on an EGFR input for activation while G_q and $G_{12/13}$ signals are refractory to inhibition of EGFR. AP-1 activation by LPA heavily depends on the G_i pathway and

accordingly is EGFR dependent. On the other hand, activation of NF-κB by LPA is mediated through an EGFR-independent G_q signaling process.

The crosstalk between RTK and GPCR in cellular functions has been a subject of extensive research in the area of signal transduction [18, 260]. Both "transactivation" and "permissive signal" models have been proposed to explain the functional dependence of GPCR signals on RTK [260]. In our studies using ovarian cancer cell lines, we did not observe strong transactivation of EGFR by LPA as reflected by only weak induction of EGFR phosphorylation (Fig. 3.5 E of Chapter 3). In addition, the effects of LPA on activation of transcription factors and the downstream gene expression were generally speaking more profound than EGF itself. Therefore, it is hard to imagine that LPA induces these biochemical and biological events through transactivation of EGFR. In contrast, our results are in concert with a permissive role of EGFR or an alternate RTK in activation of GPCR signaling. Elucidation of EGFR-dependent and EGFR-independent G protein signaling cascades and their downstream biochemical events allow us to conclude that only selective GPCR signaling pathways are regulated by EGFR.

It remains to be determined how EGFR is integrated with GPCR signaling to activate G_i and events downstream of G_i. As shown in this Chapter, again, the role of EGFR could be substituted for by activation of another RTK such as c-Met. The observation indicates that a RTK activity, not necessarily EGFR, is involved in linking GPCR to G_i activation. It has been well documented that EGFR is overexpressed or activated through mutation in many types of human cancers including ovarian cancer [244, 260]. It is conceivable that the elevated EGFR activity in cancer cells likely functions as a

default RTK to interact with GPCR signals. The basal, unstimulated activities of other RTKs may not be sufficiently high to provide such a permissive signal. However, when activated by their specific ligands, these other RTKs such as c-Met may function as alternate RTKs to interact with GPCR signals. Targeting EGFR has been an attractive approach to cancer intervention. Based on our results presented in this chapter, EGFR is essential for many biological processes evoked by GPCRs including cytokine production, cell proliferation, and cell migration and invasion. Many GPCR agonists are important mediators of these biological processes in cancer cells. Thus, inhibition of EGFR may yield therapeutic benefits from interference with GPCR signaling in addition to disconnection of EGFR from its own direct downstream effectors.

CHAPTER 5

GENERAL DISCUSSION

LPA is present in the blood at sub-micromolar concentrations [19, 20]. It is one of growth factors found in ascitic fluids of ovarian cancer patients [4, 6, 261]. Elevated plasma levels of LPA are found at both early and advanced stages of ovarian cancer, offering LPA as a potential biomarker for early detection of the malignancy [4, 5, 262]. LPA receptors are also overexpressed in primary ovarian cancer and ovarian cancer cell lines [35, 59, 263, 264]. LPA stimulates proliferation, survival and motility of ovarian and other types of cancer cells [3, 7, 29]. LPA presence in tumor microenvironment enhances aggressiveness of cancer cells at least partially through modulating expression of diverse target genes ranging from inflammatory cytokines to proteases.

Dysregulated gene expression is a hallmark of tumorigenesis. The overall aim of my project was to delineate the mechanistic details of LPA-induced gene expression in ovarian cancer cells wherein LPA has been implicated as an activating factor. Identification of major components of LPA signal transduction leading to gene expression may offer novel therapeutic targets for treatment of ovarian cancer and other human malignancies. The current work demonstrated that (i) LPA upregulates gene expression at both transcriptional and post-transcriptional levels; (ii) LPA triggers transcriptional

activation through activating major transcription factors such as AP-1, NF-κB and C/EBP; (iii) LPA activates transcription factors via RTK-dependent and independent pathways.

As described in Chapter 2, we analyzed the molecular mechanisms by which LPA stimulated expression of the Cox-2 gene in ovarian cancer cells. Cox-2 is an important inducible enzyme in the formation of prostanoids including prostaglandins, prostacyclins and thromboxanes. Targeting Cox-2 with non-steroidal anti-inflammatory drugs (NSAIDs) provides relief from symptoms of pain and inflammation [207]. Since selective inhibition of Cox-2 also reduces the risk of colon cancer [265, 266], it is generally believed that Cox-2 contributes to the pathogenesis of human cancer including ovarian cancer [267]. Therefore, it was of interest to study how the Cox-2 gene is regulated in cancer cells.

Compared to many peptide growth factors, LPA was more potent inducer of Cox-2 expression in ovarian cancer cell lines, an effect mediated by multiple LPA receptors including LPA_1, LPA_2 and LPA_5. In addition to the striking potency, another feature we observed was the prolonged duration of Cox-2 induction by LPA. LPA-induced Cox-2 expression remained highly elevated 16 hours after exposure to LPA, in sharp contrast to the transient induction of Cox-2, as an early response gene, by other agonists. Analysis of Cox-2 mRNA stability revealed that it was significantly enhanced in LPA-treated cells. We further identified HuR as an mRNA stability factor that bound to Cox-2 transcripts at 3'-UTR and protected them from degradation. Knockdown of HuR expression with siRNA attenuated the prolonged stimulation of Cox-2 by LPA, confirming an active role of HuR in sustaining Cox-2 expression in LPA-treated cells. At present, it is not known whether

LPA activates HuR through biochemical modifications or whether the association of HuR and Cox-2 mRNA is solely dependent on increased transcript levels of the latter.

An important observation made in Chapter 2 was the crucial role of C/EBP-β in transcriptional initiation of Cox-2 gene expression in response to LPA. In contrast to other studies implication of AP-1 and/or NF-κB in inducible Cox-2 expression, we observed minimal contributions of these factors to LPA-induced Cox-2 expression. In fact, expression of a dominant negative form of c-Jun (Tam67) modestly potentiated LPA-induced Cox-2 expression. The finding of the essential and unique role of C/EBP-β in driving Cox-2 expression by LPA was somewhat surprising to us as C/EBP-β had not been implicated in any biological effects of LPA. However, our results from multiple approaches including promoter analysis, ChIP assay, and expression of dominant negative of C/EBP-β, LIP all pointed to critical involvement of C/EBP-β in activation of the Cox-2 gene promoter in LPA-stimulated cells.

In Chapter 3, we extended to investigate the molecular mechanism for activation of C/EBP-β by LPA and the general role of this transcription factor in LPA-mediated gene expression. We focused on the C/EBP-β isoform because it was abundantly expressed in ovarian cancer and correlated with aggressiveness of the disease [135]. We showed that LPA induced a rapid phosphorylation of C/EBP-β and its DNA-binding and transcriptional activities. The C/EBP-β phosphorylation is one of the major biochemical events associated with its activation [121, 125, 126]. Since the C/EBP-β phosphorylation correlated well with DNA-binding and transcriptional activities in LPA treated cells, it is likely that LPA-

induced phosphorylation represent a key step leading to functional activation of the transcription factor although the impacts of LPA on other biochemical changes associated with C/EBP-β activation, such as ubiquitination, sumoylation and acetylation, are yet to be assessed and therefore should be a focus of future studies.

Some mammalian cells carry C/EBP-β in the cytosol and nucleus and activation of C/EBP-β commensurate with an increased nuclear translocation of the protein [142, 268]. In ovarian cancer cells, however, C/EBP-β was exclusively seen in the nuclei and LPA treatment did not change such a distribution pattern. Thus, nuclear translocation is not a mechanism to activate transcriptional capacity of C/EBP-β. In addition, the kinase that activates nuclear C/EBP-β upon LPA stimulation has not been identified. Considering the role of C/EBP-β in the induction of Cox-2 expression by LPA, it is unlikely that nuclear C/EBP-β kinase would be of MAPK lineage given that inhibition of MAPK cascade did not block LPA-induced Cox-2 expression. The detail of that unique LPA GPCR-activated pathway that bypasses MAPK signaling components may be critical not only for the identification of nuclear C/EBP-β kinase but perhaps for the discovery of more upstream targets of Cox-2 activity. Moreover, the export of C/EBP-β out of the nucleus by nuclear exportins has been shown to result in repression of certain genes. Thus, temporal manipulation of C/EBP-β levels via nuclear export may be an attractive therapeutic option.

Overexpression of LAP was not sufficient to increase C/EBP-β transcriptional activity strongly suggesting that LPA-mediated CEBP-β activation is due to biochemical modification of C/EBP-β rather than in an increase in overall CEBP-β protein levels. In

extended stimulation conditions, activation and expression of C/EBP-β-target genes may continue to benefit from continual presence of LPA, such as is found in the ascites of ovarian cancer patients. By extending our study to other LPA-target genes, we demonstrated that, in addition to Cox-2, C/EBP-β was also involved in LPA induction of inflammatory cytokine IL-6 and metastatic factor uPA, underscoring a general role of CEBP-β in modulation of multiple LPA target genes.

A highlight of the work described in Chapter 3 is the finding of the dependence of C/EBP-β activation on crosstalk between LPA GPCRs and EGFR. The effects of LPA on CEBP-β phosphorylation, DNA-binding and transcriptional activities and the ultimate induction of Cox-2 were all sensitivity to inhibition of EGFR kinase activity with AG1478. LPA-induced IL-6 and uPA gene expression was also impaired by inhibition of EGFR, further substantiating C/EBP-β as rate-limiting factor in LPA-induced Cox-2, IL-6 and uPA expression. It is interesting to note that the dependence on EGFR could be overcome by activation of another RTK c-Met. In recovery experiments, costimulation with HGF efficiently revoked the suppression imposed by EGFR inhibition. This observation suggests that a receptor tyrosine kinase activity, not specifically EGFR, is required for LPA-induced activation of C/EBP-β and expression of the downstream targets. The reason that EGFR plays such a default role is most likely due to the fact that the basal EGFR activity is more prominent than other RTKs, particularly in malignant cells where EGFR is commonly overexpressed and activated through mutations [244, 260]. Another interesting question is whether the basal, unstimulated activity of EGFR in ovarian cancer cells is

sufficient to provide such as permissive signal for GPCR activation of CEBP-β. Alternatively, EGFR could be "transactivated" during LPA stimulation. However, we observed only weak activation of EGFR by LPA compared to the marked effect of EGF itself in ovarian cancer cell lines. It is difficult to fit our observations into the "transactivation" model given the fact that full activation of EGFR by EGF only triggers weak activation of C/EBP-β and Cox-2 expression. Therefore our results are more compatible with the hypothesis that a basal EGFR activity is necessary for transmission of GPCR signaling to C/EBP-β activation.

To further characterize the crosstalk between GPCR and RTK in activation of transcription factors, we next asked whether the RTK signal is a general requirement for activation of other transcription factors by LPA. As described in Chapter 4, we examined LPA-induced activation of AP-1 and NF-κB in ovarian cancer cell lines. Interestingly, AP-1 activation in response to LPA indeed relied on EGFR activity analogous to LPA-induced C/EBP-β activation. However, LPA stimulated NF-κB activation independently of EGFR activity. Hence, the intact EGFR is differentially required for activation of these prominent transcription factors. NF-κB activation by LPA offers an excellent readout of EGFR-independent signals downstream of LPA receptors. It however leaves with us a quest to identify more EGFR-independent transcription factors and genes. Such molecules may be involved in unknown mechanisms through which ovarian tumor cells exhibit resistance to EGFR therapy [269]. A microarray analysis of LPA-induced transcriptome in cells pretreated with EGFR inhibitors would be an excellent methodology through which such informative profile may be obtained.

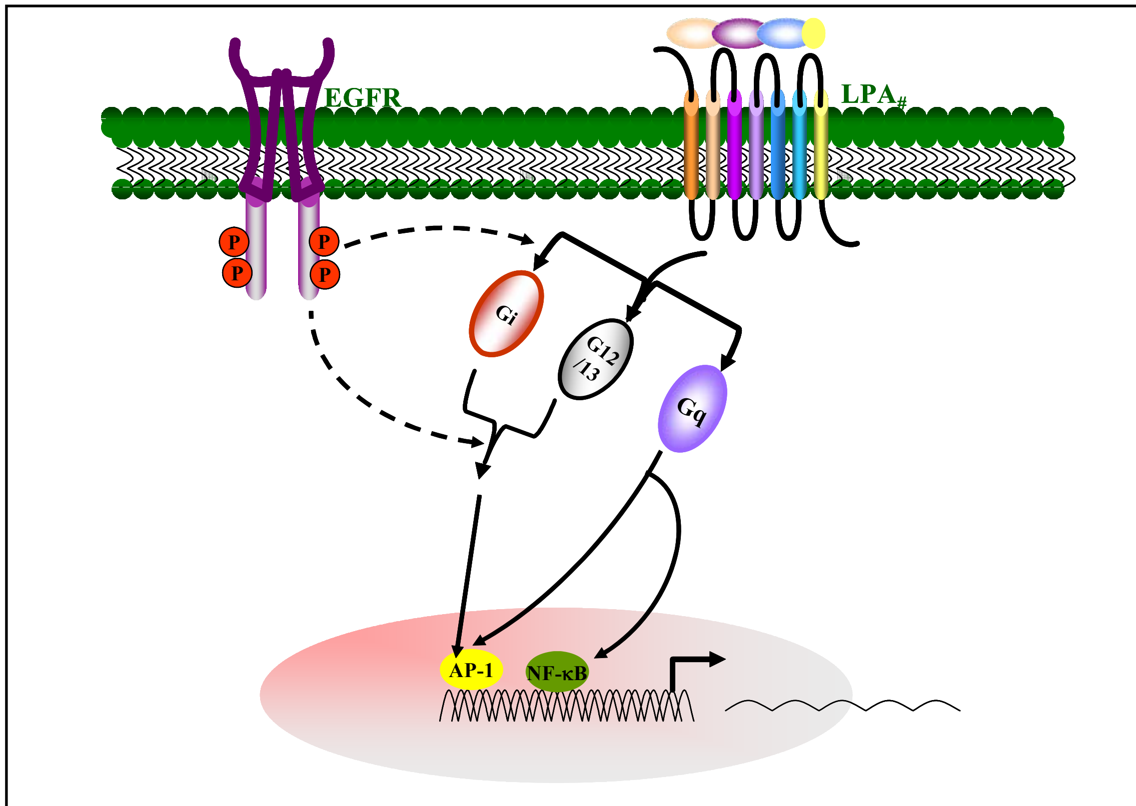


Fig. 5.1. **Hypothetical model of activation of G proteins and transcription factors by LPA.**
Obligatory inputs from basal EGFR activity may feed into signals downstream of LPA receptors at diverse points, particular before and/or after activation of G_i. Activation of AP-1 by LPA is mediated by activation of multiple G proteins. However, NF-κB is activated downstream of G_q signaling cascade.

Molecular and pharmacological approaches demonstrated that activation of AP-1 and NF-κB were mediated by different G protein signaling cascades downstream of LPA receptors. The EGFR-independent G_q pathway was a mediator of NF-κB while the EGFR-dependent Gi signaling cascade was the predominant and most critical player in LPA-induced activation of AP-1. These results presented in Chapter 4 provide in-depth insights

into the role of EGFR in activation of intracellular G proteins and their downstream molecules.

The hypothetical model depicted by Fig. 5.1 simplifies the current status of this project as well as future prospects. It will be of particular interest to elucidate how EGFR activity is involved in activation of G_i in cooperation with GPCR. It is also possible that EGFR is required for activation of a G_i effector that is located downstream of G_i but upstream of Ras. Another direction is to find the link between G_i and C/EBP-β. Based upon the observation that both G_i and C/EBP-β activation rely on EGFR activity, we predict that phosphorylation of C/EBP-β lies downstream of G_i. We have already assessed a number of Gi-mediated signals including the MAPK pathways. We have unfortunately not confirmed the connection yet.

The G_i signaling is essential for many biological responses to LPA and other GPCR ligands. We observed significant but incomplete inhibition of LPA-induced cell proliferation, migration and invasion by EGFR inhibitors. These results suggest that EGFR-independent, G_q or $G_{12/13}$-medaited signals could be operational partially mediating these responses. Thus, simultaneous targeting EGFR and LPA receptors seems to be an appealing approach to inhibit proliferation, invasion and metastasis of ovarian cancer cells. This could be evaluated both in vitro and in vivo.

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PUBLICATIONS

- **Oyesanya RA**, Fang X Differential Requirement of Basal Tyrosine Kinase Activity in LPA-Induced Transcription factors Activation *In Preparation*
- **Oyesanya RA**, Lee ZP, Wu J, Chen J, Song Y, Mukherjee A, Dent P, Kordula T, Zhou H, Fang X Transcriptional and post-transcriptional mechanisms for lysophosphatidic acid-induced cyclooxygenase-2 expression by in ovarian carcinoma cells, *FASEB J* 2008 Aug;22(8):2639-51
- Song Y, Wu J, **Oyesanya RA**, Lee ZP, Mukherjee A, , Fang X Lysophosphatidic acid induces multiple isoforms of vascular endothelial growth factor in ovarian cancer cells through c-Myc and Sp-1 transcription factors. Clin Cancer Res. 2009 Jan 15;15(2):492-501.
- Song Y, Wilkins P, Hu W, Murthy KS, Chen J, Lee Z, **Oyesanya R**, Wu J, Barbour SE, Fang X Inhibition of calcium-independent phospholipase A2 suppresses proliferation and tumorigenicity of ovarian carcinoma cells *Biochem J. 2007 406(3):427-36*

AWARDS & HONORS

- Prestigious Herbert Evans Award for Excellence in Biochemistry

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- Phi Kappa Phi Honor Society

  Graduate School Scholarship & Membership

  May, 2008 · 2008 Recipient
- CC Clayton Award for Academic & Research Excellence

  May, 2007 · 2006 Recipient
- Excellence in Biochemistry Award

  May, 2007 · Forbes Day Seminar Presentation
- NIH Travel award, 2006 Southeastern Regional Lipid Conference

  November, 2006 · Short Seminar Presentation
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CONFERENCES AND SYMPOSIA ATTENDED

- Daniel Watts Day Symposium

  October, 2008 · Poster Presentation
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- Integrated Cellular and Molecular Signaling (ICAMS) Research Retreat

  October, 2006 · Poster Presentation
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