CHARACTERIZATION OF MICROSOMAL PROSTAGLANDIN E SYNTHASE-1 GENE REGULATION BY THE PRO-INFLAMMATORY CYTOKINE INTERLEUKIN 1-BETA

By

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To my mother Edith and my husband Cory

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ALLN	Calpain inhibitor *N*-acetyl-leucyl-leucyl-norleucinal
BAL	Bronchial alveolar lavage fluid
CaMKII	Calcium and calmodulin-dependent protein kinase II
C/EBP	CCAAT-enhancer binding protein
ChIP	Chromatin immunoprecipitation
COX	Cyclooxygenase -1or -2
COXIB	Cyclooxygenase-2 selective inhibitor
DP	Prostaglandin D_2 receptor
ECM	Extracellular matrix
EGR1	Early growth response factor-1
EP	Prostaglandin E_2 receptor
ERK	Extracellular signal-regulated kinase
FLAP	5-Lipoxygenase activating protein
JNK	c-Jun N-terminal kinase
HETE	Hydroxyeicosatetraenoic acid
HODE	hydroxyoctadecadienoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
hGH	Human growth hormone
hnRNA	Heterogeneous nuclear RNA
HS	Hypersensitive site
IL-1β	Interleukin-1β
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase

MEF	Mouse embryonic fibroblasts
MSK	Mitogen- and stress-activated protein kinase
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID	Nonsteroidal anti-inflammatory drugs
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator-activating receptor
PGD	Prostaglandin D_2
PGE	Prostaglandin E_2
PGH	Prostaglandin H_2
PGES	Prostaglandin E synthase
PLA	Phospholipase
TK	Thymidine kinase
TNFα	Tumor necrosis factor α
TxA_2	Thromboxane

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The arachidonic acid (AA) pathway is a major contributor to the inflammatory response, pain production and cellular homeostasis. AA is liberated from membrane phospholipids by cytosolic PLA2alpha (cPLA2alpha) activity and then metabolized by either the cyclooxygenase (COX) or lipoxygenase (LOX) enzymes. The COX enzymes regulate the production of downstream prostanoids known to be involved in the regulation of a number of biological and pathophysiological processes. Of these prostanoids, PGE2 is the most widely studied due to its key role in inflammation. Over the years, the study of PGE2 biosynthesis and regulation focused entirely on the role of COX-2. More recently, the trend has shifted towards understanding the role of specific PGE2 terminal synthases. There are five known PGE synthases and microsomal PGES-1 (mPGES-1) has emerged as the crucial enzyme responsible for PGE2 production. mPGES-1 is highly induced by pro-inflammatory cytokines and existing gene regulation studies highlight the importance of early growth response factor-1 as a key regulator of mPGES-1 expression. This study demonstrates that mPGES-1 is induced by interleukin 1-beta (IL-1beta) in pulmonary fibroblasts requiring *de novo* transcription and identifies a hypersensitive site (HS) within the distal promoter region that exhibits both basal and inducible enhancer activity. Functional analysis of HS led to the identification of a binding site for CCAAT/enhancer binding

protein within the enhancer and illustrated the importance of this element in recapitulating the complete cytokine induction of mPGES-1 gene expression.

cPLA2alpha is activated by intracellular calcium levels and kinase activity but the exact signaling mechanism involved is still unclear. This study attempted to identify key factors involved in the IL-1β induction of cPLA2alpha in pulmonary cells. The results introduce a feed forward mechanism involving the initial rapid induction of cPLA2alpha enzymatic activity and the involvement of a downstream AA metabolite, 15-LOX as being necessary for the cytokine-mediated induction of cPLA2alpha.

mPGES-1 expression is highly up-regulated in breast cancer and recent studies demonstrate a role for estrogen and possibly TNFalpha in mediating mPGES-1 gene expression. The final study explores the involvement of TNFalpha and illustrates that it is a potential mediator of mPGES-1 gene expression in breast cancer.

CHAPTER 1
INTRODUCTION

Overview of the Arachidonic Acid Pathway and Metabolites

Redness, pain, swelling, these are all visible characteristics of the inflammatory response, but at the cellular level the response is much more complicated. In the initial stages, arachidonic acid is metabolized to form eicosanoids which include prostaglandins, leukotrienes, hydroxyeicostetraenoic acid and lipoxins as illustrated in Figure 1-1 (1-3). The eicosanoids then serve as signaling molecules, regulating a variety of processes including chemotaxis (4), vasodilatation (5), pain (6), fever (7), anaphylaxis and vasoconstriction (8,9). Aside from the inflammatory response, eicosanoids have also been implicated in a number of disease states including inflammatory bowel disease/Crohns' disease, many cancers such as breast, colon, prostate and lung cancer, rheumatoid arthritis, chronic obstructive pulmonary disease (COPD) and cardiovascular disease (3,8,10-16).

Prostaglandin/Prostanoid

Prostanoids were initially discovered when an extract of sheep seminal fluid was incubated with arachidonic acid (17). The first steps of prostanoid synthesis involve the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) by the action of prostaglandin endoperoxide H synthase also called cyclooxygenase (COX-1 and COX-2). PGH_2 serves as the central intermediate and substrate for the synthesis of the following prostanoids - PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TxA_2 (18,19). The conversion of PGH_2 to the various prostanoids is mediated by the action of specific terminal synthases which function not only in the catalysis of these reactions but also in the regulation of prostanoid expression (1,11,20).

Prostanoids have been shown to regulate a wide variety of complex processes including inflammatory responses, female reproduction, tumorigenesis, vascular hypertension, kidney

function, gastric mucosal protection, pain sensitivity, vasodilatation, bronchoconstriction, pyresis, parturition, sleep and many diseases within the body (21-24). PGD_2, for instance, plays a role in asthma, smooth muscle relaxation, the activation of eosinophils and is synthesized by mast cells in the lungs following allergen challenge and has been thought to play a role in asthma, as well as, in the activation of eosinophils (5,25,26). PGD_2 along with PGE_2 are also necessary during the sleep wake cycle; PGD_2 promoting sleep and PGE_2 promoting wakefulness (27,28). On the other hand, TxA_2, is synthesized by platelets, functions in platelet aggregation and vasoconstriction in the cardiovascular system (29,30). Conversely, PGI_2 acts opposite to TxA_2 as an anticoagulator for platelets, a vasodilator and like $PGF_{2\alpha}$ has been widely studied in pregnancy during embryo implantation (31-33). PGE_2 has been implicated to promote fever, inflammation, vasodilatation, cancer, pain and is involved in reproductive processes (11,34-41). $PGF_{2\alpha}$ also plays a critical role in reproductive processes promoting myometrial contractions, cervical relaxation and ovulation (42,43).

Arachidonic acid metabolites play a role in overall lung health and function, the predominant forms being PGD_2,PGE_2, and TxA_2 (44). Airway inflammation, airway obstruction, remodeling, hypertrophy/hyperplasia of bronchial smooth muscle cells and eosinophil infiltration are all principal features of asthma (45-47). PDG_2 is a potent bronchoconstrictor that is produced by mast cells and purported to play a role in allergen-induced asthma (48,49). In 2000, Matsuoaka et al. (25) highlighted the role of PGD_2 in allergic asthma. Using PGD_2 receptor (DP) null mice, they were able to show that both the wild type and mutant mice had similar levels of serum IgE, the antibody produced in response to antigen-induced asthma. Furthermore, after sensitization and aerosolized application of ovalbumin, in a model of allergen-induced asthma, wild type mice exhibited increased infiltration of eosinophils and

lymphocytes, while the DP -/- mice showed only marginal increases in the number of these infiltrated cells. Also the wild type animals showed an increase in airway hyperactivity compared to the DP -/- mice as well as an increase in the production of T_H2 cytokines. Overall this study provided strong evidence of the role of PGD_2 in mediating the asthmatic response (25).

Derived from platelets, TxA_2 is a known to be a constrictor of bronchial smooth muscles and a stimulator of airway smooth muscle cell proliferation (29,44). Asthmatic patients are known to produce excessive amounts of TxA_2, as measured in their urine (50), bronchoalveolar lavage (BAL) fluid (51) or exhaled air condensate (52). Davi et al. (53) illustrated that patients suffering from chronic obstructive pulmonary disease showed increased urinary excretion of TxA_2 versus healthy patients and that hypoxia may stimulate increased synthesis of TxA_2.

The most potent prostanoid in the human body is PGE_2 and within the lung it is bronchoprotective (15). A variety of cell types contribute to PGE_2 production including macrophages, dendritic cells and lung fibroblasts (15,54). In response to pro-inflammatory mediators and stimuli such as IL-1β, LPS and phorbol esters, human alveolar macrophages, lung fibroblasts and airway epithelial cells up-regulate COX-2 expression which alternatively leads to an increase in PGE_2 levels (55-57). Normal lung cells produce collagen, elastin, cytokines/growth factors and extracellular matrix (ECM) proteins which provide structural integrity as well as shape, movement, growth and differentiation. In response to injury or environmental cues, fibroblast proliferation is activated and there is an increase in collagen synthesis/ECM deposition which if left unchecked leads to fibrosis (15,39). Over the years, several studies have shown that PGE_2 is known to inhibit fibroblast migration, proliferation, collagen synthesis and eosinophil degranulation, thus highlighting the protective effects of PGE_2

in the lung (58-60). A detailed description of PGE_2 and the synthase responsible for its biosynthesis, which is a central topic of this dissertation, will follow.

An alternative metabolic pathway for arachidonic acid involves the synthesis of cysteinyl leukotrienes, leukotriene C4, -D4 and -E4 (LTC4, LTD4 and LTE4) by the action of 5-lipoxygenase (5-LOX) and leukotriene C4 synthase. These leukotrienes are produced by leukocytes, lung fibroblasts, platelets and endothelial cells (61-63). In the lung, cysteinyl leukotrienes are chemoattractants and are involved in fibroblast proliferation and collagen synthesis (64,65). Over the years, cysteinyl leukotrienes have been implicated in lung disease, particularly asthma and have been the target for drug development and treatments (66-69). Further, the role of leukotrienes in pulmonary function was evaluated in animal models of fibrosis (68). In bleomycin-induced fibrosis, 5-LOX -/- mice exhibited decreased levels of ECM proteins and a reduction in the recruitment of immune cells (lymphocytes, eosinophils and macrophages) compared to wild type mice. The 5-LOX -/- mice also showed increased PGE_2 production after bleomycin induction, which may explain the reduced response to bleomycin-induced inflammation (70).

Together these studies highlight the importance of eicosanoids as they relate to lung health and function. Each metabolite represents a potential therapeutic target for the development of new drugs used for the treatment of asthma, fibrosis and other pulmonary disorders and potentially as therapies in lung cancer. These studies also reveal the diversity exhibited by the lung, thus establishing it as an interesting model for gene regulation studies.

Cytosolic Phospholipase $A_2\alpha$ ($cPLA_2\alpha$) and Arachidonic Acid Metabolism

In the 1930's essential fatty acids were discovered as compounds that were vital for human health but could only be obtained through diet (41,71). There are two main families of essential fatty acids, omega-3 containing alpha-linolenic acid and omega-6 containing linoleic acid, which

serve as the starting point for the production of polyunsaturated fatty acids including arachidonic acid (2,13,41). Arachidonic acid, an omega-6 fatty acid, is generated by the hydrolysis of phospholipids via the action of phospholipase A_2s (PLA_2) (2,13,41,72-77). There are five categories of phoshpolipases: secreted phospholipases (78-81), group (IV) cytosolic PLA_2 (82-84) and intracellular group (VI) calcium-independent PLA_2 (85,86), PAF acetylhydrolysases and lysosomal PLA_2s (87-89). While each class of PLA_2 is capable of cleaving arachidonic acid from phospholipids, group IV PLA_2, particularly cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$), shows a high specificity for cleavage of arachidonic acid from the sn2 position of glycerophospholipids and this reaction is illustrated in Figure 1-2 (82,84,90). The newly synthesized arachidonic acid is further metabolized to leukotrienes, lipoxins, prostaglandins and hydroxyeicostetraenoic acids, as shown in Figure 1-1.

Regulation and Activation of Cytosolic $PLA_2\alpha$

Group (IV) cytosolic PLA_2 contains six isozymes, $cPLA_2\alpha$, $cPLA_2\beta$, $cPLA_2\gamma$, $cPLA_2\delta$, $cPLA_2\varepsilon$ and $cPLA_2\zeta$, which all share a catalytic dyad and a homologous C2 domain involved in Ca^{2+}-dependent phospholipid binding. The only exception to this group is $cPLA_2\gamma$; which lacks the C2 domain but is isoprenylated at its C-terminus and thereby thought to be membrane-associated (91,92). Localized to chromosome 1q25, $cPLA_2\alpha$ is ubiquitously expressed in all human tissues, with an elevated basal level in the lung. The enzymatic activity and levels are also induced in response to pro-inflammatory stimuli and various growth factors (54,93-100). Furthermore, use of IL-4 or glucocorticoids has been shown to inhibit $cPLA_2\alpha$ activation and thus downstream eicosanoid formation (99,101-103).

Numerous studies have evaluated the enzymatic activity of $cPLA_2\alpha$ in terms of arachidonic acid production and found that the protein is also regulated at the post-translation level. The activity of the 85kD enzyme is known to be induced by phosphorylation of a serine residue at

position 505, mediated by mitogen activated protein kinases (MAPK) (104,105). Two other serine residues, at position 515 and 727 have also been shown to be important for cPLA$_2$α enzyme activity. Ser515 is reportedly phosphorylated by calcium/calmodulin-dependent kinase II (CaMKII) and Ser727 by mitogen-activated protein kinase interacting kinase (MNK-1) (93,106-108) and mutation of either of these two residues results in a loss of cPLA$_2$α activity. Previous reports have shown that micromolar levels of intracellular calcium levels promote the translocation of cPLA$_2$α from the cytoplasm to the nuclear envelope and endoplasmic reticulum, putting the enzyme in close proximity to its substrate and other enzymes in the arachidonate pathway (109-112). Aside from intracellular calcium levels, full activation of cPLA$_2$α, is dependent on the activity of members of the MAPK pathway including MKK3/MKK6 and p38 which will be discussed in some detail in Chapter 5.

In different cells and under certain conditions cPLA$_2$α can localize to different regions such as the nucleoplasm in endothelial cells, the plasma membrane in neutrophils and lipid bodies in macrophages, mast cells, neutrophils and fibroblasts (113-115). As further verification of the role of calcium in cPLA$_2$α, the use of calcium agonists were employed and shown to activate cPLA$_2$α activity leading to translocation and the increased release of arachidonic acid (116).

The physiological importance of cPLA$_2$α has been highlighted by pathological studies of cPLA$_2$α-deficient mice. Overall these mice appear to develop normally, however they do exhibit a few abnormalities, including a reduced litter size (117), impaired parturition (118), kidney problems (urine-concentrating defect, aquaporin 1 defect/diminished water reabsorption) (119) and the propensity to develop ulcerated intestines (120). In disease state models for lung injury (111), such as experimental autoimmune encephalomyelitis (121), MPTP (1-methyl 4-phenyl

1,2,3,6-tetrahydropyridine) neurotoxicity/Parkinsonian disease (122), ischemic brain injury (118), collagen induced arthritis (123), atherogenesis (124); $cPLA_2\alpha$(-/-) mice show a decreased incidence and severity of the respective diseases in comparison to their wild-type counterparts. Furthermore, peritoneal macrophages isolated from the mutant mice show reduced levels or loss of arachidonic acid release and downstream eicosanoid signaling (118,125).

Lipoxygenases (LOX)

The LOX enzymes form another major pathway involved in both arachidonic acid and polyunsaturated fatty acid metabolism. Lipoxygenases reduce fatty acid substrates by oxygenation, leading to the formation of hydroperoxyeicosatetraenoic acid (HPETE), hydroxyeicosatetraenoic acid (HETE), leukotrienes, lipoxins or hydroxyoctadecadienoic acid (HODE). There are four LOX enzymes, 5-, 8-, 12- and 15-, classified according to the site of oxygen insertion within arachidonic acid (126-128). The LOX enzymes are very similar in both mice and humans; in mice there are seven forms of LOX enzymes, four 12-LOX, 8-LOX, 5-LOX, e-LOX1 (non-expressed epidermal) most of which map to chromosome 11 while in humans there are four forms, 5-LOX, 12-LOX, 15-LOX1 and 15-LOX2, three of which are localized to chromosome 17 (129-132).

Various cell types such as leukocytes, macrophages, granulocytes, dendritic and mast cells express 5-LOX. An approximately 75kD protein, 5-LOX catalyzes the formation of 5-HPETE leading to the formation leukotrienes. 5-LOX expression and activity has been observed in bronchial asthma, cardiovascular disease and various cancers (67,131,133-135). There are two isoforms of 15-LOX in humans, 15-LOX1 and 15-LOX2, which are 78kD proteins. 15-LOX1 is closely related to the murine leukocyte 12-LOX and 15-LOX2 is similar to murine 8-LOX. Both 15-LOXs utilize arachidonic acid as a substrate; 15-LOX2 preferentially converts arachidonic acid to 15S-HETE while 15-LOX1 produces 15S-HETE and 12S-HETE (132,136,137). Aside

from arachidonic acid, it is well documented that 15-LOX1 metabolizes linoleic acid to 13(S)-HODE (138-140). 15-LOX1 is found in airway epithelium, monocytes, prostate and colorectal carcinomas while 15-LOX2 is expressed in the cornea, skin, hair root, lungs and prostate gland (132,141,142). The importance of 15-LOX2 to cPLA$_2$α gene regulation will be addressed in Chapter 5 where data will be presented on a feed forward mechanism controlling IL-1β-dependent induction of cPLA$_2$α.

Cyclooxygenases (COX)

After arachidonic acid is liberated from glycerophospholipids by PLA$_2$ activity, it is then metabolized to prostaglandin G$_2$ then prostaglandin H$_2$ in a series of oxygenation reactions catalyzed by cyclooxygenases (COX). Currently there are three known COX isoforms, COX-1, COX-2 and COX-3 (which is a splice variant of COX-1 and also referred to as COX-1b) (143,144). COX-1 is constitutively expressed in most tissues and cell types, and is known to be important in development. COX-1 expression is induced by phorbol esters in monocytes, megakaryoblasts, endothelial cells and fibroblasts by IL-1β and TGF-β (145-148). COX-3 is a splice variant of COX-1 and although its precise role has not yet been determined, it is found to be highly expressed in the cerebral cortex and heart (144). Where COX-1 is constitutively expressed, COX-2 is inducibly expressed in many tissues in response to growth factors, cytokines IL-1β, TNF-α, LPS and phorbol esters (149-151).

The two main COX isoforms, COX-1 and COX-2 are expressed in the lung and are known to be involved in the ultimate production of PGE$_2$. Figure 1-3 illustrates the COX pathway leading to the production of PGE$_2$. The availability of COX null mice has allowed for a clearer understanding of the roles these enzymes play in lung health. Knockout models for COX-1 and COX-2 have revealed that COX-2 expression is required for PGE$_2$ production (152). In a recent study using either COX-1 -/- or COX-2 -/- mice, the results illustrated that following allergen

exposure, mice deficient for either COX isoform showed an increase in airway infiltrates and exhibited severe inflammation in the lungs compared to wild type mice, presumably due to reduced levels of PGE_2 (153). Also, allergen-induced COX-2 -/- mice showed increased airway responsiveness when exposed to metacholine versus wild type mice and a greater production of BAL cells and proteins (153). In a somewhat similar study, Zeldin et al. (154) showed that mice deficient for either COX-1 -/- or COX-2 -/- exposed to aerosolized LPS had increased bronchoconstriction with no difference in the number of BAL cells or lung histopathology as compared to wild type mice. However, they did observe reduced levels of BAL cytokines/chemokines and PGE_2, which lends further credence to the importance of the COX isoforms in PGE_2 production and lung health.

Molecular and Transcriptional Regulation of COX-2 Expression

Although both COX-1 and COX-2 are involved in the production of downstream prostanoids, COX-2 transcriptional regulation has been extensively studied. The gene encoding COX-2 is located on chromosome 1 spanning 8.3 kb and contains 10 exons. The COX enzymes are structurally similar, sharing ~61% homology at the amino acid level (143,155). They differ only slightly in their catalytic sites, where position 523 in COX-1 is an isoleucine residue and the analogous residue in COX-2 is a valine. This difference allows for the formation of a side pocket which is known to be critical for specific COX-2 inhibition (143). COX-2 encodes a 4.6 kb full length transcript and a 2.8 kb polyadenylated variant. The 3'UTR of COX-2 contains an instability element that is involved in its post-transcriptional regulation (155). The promoter region of COX-2 contains a number of transcription factor binding sites including NF-κB, C/EBP, cyclic AMP response elements, a TATA box and an E-box (155-157). COX-2 expression is also known to be influenced by chromatin remodeling as p300 plays a role in transcriptional activation of COX-2 (158,159).

In human umbilical vein endothelial cells, human foreskin fibroblasts and human airway smooth muscle cells, COX-2 mRNA and protein expression is up-regulated in response to IL-1β and mediated by NF-κB and C/EBP (160,161). Also, LPS has been shown to induce COX-2 expression in RAW 264.7 cells mediated by CRE-1, C/EBP and NF-κB (162). ERK1/2, p38MAPK and JNK pathways have also been identified as having a role in COX-2 expression (163,164). It should be noted that up-regulation of COX-2 expression also leads to increased prostaglandin synthesis and deregulation of COX-2 expression is associated with inflammatory diseases and many cancers making COX-2 an attractive target for pharmacological inhibitors.

Inhibition of COX Activity by Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

In the early 1970's NSAIDs such as aspirin, ibuprofen and indomethacin were found to inhibit both COX-1 and COX-2 activity and prostaglandin production. Aspirin is capable of inhibiting both COX-1 and COX-2, but it is known to selectively acetylate Ser530 on COX-1, blocking the active site channel and irreversibly inhibiting COX-1 activity (165,166). Since that time, numerous COX-2 specific inhibitors (COXIBs) have been developed along with drug trials to assess their potential anti-inflammatory and analgesic effects (167). While COXIBs such as celecoxib, valdecoxib and rofecoxib offered new hope in the management of cancer and the treatment of rheumatoid arthritis as an alternative to the use of traditional NSAIDs, their efficacy has been questioned due to the appearance of life-threatening side effects. It was found that prolonged use of COXIBs was associated with increased risk of gastrointestinal and cardiovascular problems (168-170). In a 1994 study by Garcia Rodriguez et al. (171), they reported that patients taking NSAIDs, particularly piroxicam, exhibited an increase risk of gastrointestinal complications. Alternatively, a 2001 study by Teismann and Forger (172), reported the protective effects of COX-2 inhibition on Parkinson's disease. In general, there have been numerous reports on both the protective and adverse effects associated with COX

inhibition. While COX-2 represents an attractive target for pharmacological inhibitors due to its aberrant expression in chronic diseases, the use of COXIBs is still somewhat controversial as are the downstream effects on prostanoid synthesis; therefore further studies and clinical trials are needed to fully understand the off-target effects of these inhibitors. Furthermore, the development of specific inhibitors of downstream synthases may offer equally beneficial outcomes with reduced side effects. The studies in chapter 3 will address the regulation of a specific PGE_2-dependent synthase that offers such an alternative therapeutic target.

Prostaglandin E_2 (PGE_2)

Of all the prostanoids listed thus far, PGE_2 is by far the most widely studied, as it plays a role in a number of inflammatory conditions and biological processes. PGE_2 exerts it effects through binding to one of four receptors, EP1, EP2, EP3 or EP4 (173,174). Each receptor is expressed in specific tissues of the body, for instance EP2 is expressed in the lung, small intestine, thymus, uterus and kidneys (175), while EP1 is expressed in the breast, stomach and the skin (176). From the early studies conducted on metabolites of arachidonic acid, a link between COX-2 and PGE_2 was identified, wherein it was shown that after treatment with inflammatory stimuli such as cytokines, growth factors and oncogenes an increase in COX-2 levels led to a subsequent increase in PGE_2 production (57,177). In many cell culture models, increases in COX-2 expression were shown to correlate with increases in PGE_2 production (57). As previously discussed, NSAIDS, including indomethacin, ibuprofen, piroxicam and sulindac along with aspirin are known COX inhibitors (178,179), competitively inhibiting the synthesis of PGG and PGH_2 by the COX enzymes. Aspirin therapy is known to be beneficial to those suffering inflammation, pain and cardiovascular health, but one of the side effects is gastrointestinal complications (169,170). In general, when COX-2 levels are inhibited by

treatment with NSAIDS, glucocorticoids or COX-specific inhibitors, PGE_2 levels were also reduced.

Apart from regulation by COX-2 and the EP receptors, PGE_2 production is dependent on specific PGES synthases while its metabolism is dependent on the cytosolic enzyme, hydroxyprostaglandin dehydrogenase (15-PGDH) (180,181). An NADP dependent enzyme, 15-PGDH catabolizes the oxidation of prostaglandins to the 15-keto form thereby reducing their biological activity. In a recent study, Yan et al. (181) showed that 15-PGDH also functions as an antagonist in colorectal cancer. They found that 15-PGDH expression is greatly reduced in colon cancer compared to normal colon mucosa. Subsequent addition of 15-PGDH and treatment with the growth factor TGF-β restored 15-PGDH expression and tumor suppression.

PGE_2 Activity is Regulated by EP Receptors: Evaluation of EP-Receptor Knockout Mice

Considering the fact that PGE_2 activity is dependent on the levels of each EP receptor, numerous studies have been conducted on individual receptor knockout animals. Knockout animals for EP1, EP2 or EP3 have been generated; EP4 has also been generated but most of these animals die during the neonatal period (182). The EP3 receptor knockout has been investigated during the febrile response (183). While EP1 and EP2 knockout animals are shown to exhibit a fever when PGE_2 is administered, EP3 -/- mice show no signs of a fever after administration of PGE_2, LPS or IL-1β (35,183,184). However, only after stress or stimulus-hyperthermia, do the animals exhibit a febrile response (35,183-185).

In the case of the EP1 receptor, EP1 -/- mice have been studied in pain perception and blood pressure models (38,186). Compared to wild-type animals, the EP1 -/- mice show a reduced sensitivity to pain and there is a significant change in their cardiovascular profile (38,186-189). Like the EP1 receptor knockout, the EP2 -/- mice have also been investigated in regard to blood pressure and reproduction. It was found that when fed a normal diet, blood

pressure was reduced in EP2 -/- mice compared to wild-type mice, but on a high salt diet EP2 -/- mice had a significant increase in blood pressure (189). With regard to reproduction, while EP2 -/- mice were no different than wild-type mice phenotypically, EP2 -/- mice exhibited reduced pregnancy rates and delivered smaller litters compared to wild-type mice (37,188,190,191). Overall, the EP receptor knockout studies highlight the importance of PGE_2 in a number of biological functions including cardiovascular homeostasis, reproduction, renal activity, fever and pain perception.

Prostaglandin E Synthase (PGES)

As the contribution of PGE_2 to many biological processes continues to be investigated, the focus of PGE_2 production has shifted to studying the role of the PGE_2 specific synthases. Jakobsson et al. (192) were the first to clone and characterize a human PGES, showing that this enzyme was part of the MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) family of proteins and that it was capable of catalyzing the terminal step conversion of PGH_2 into PGE_2. Their over-expression data revealed that PGES was a membrane protein, which, when incubated with glutathione and PGH_2 showed high levels of PGES activity (192). Earlier work by the same group and others revealed two key residues, arginine at position 100 and tyrosine at position 130, conserved within the MAPEG family, that are essential for enzymatic activity (193-196). When the arginine residue was mutated this resulted in a loss enzymatic activity.

There exist five forms of PGES, two membrane or microsomal prostaglandin synthases (mPGES-1 and mPGES-2), cytosolic prostaglandin synthase (cPGES/p23) and two glutathione transferases (GSTM2-2 and GSTM3-3) (197,198). In terms of activity, mPGES-2 is glutathione independent, constitutively expressed and known to associate with both COX-1 and -2, while cPGES is functionally coupled to COX-1, mPGES-1 is glutathione-dependent and functionally

coupled to COX-2. Although all forms of PGES contribute to the overall production of PGE_2, mPGES-1 is strongly up-regulated in response to pro-inflammatory stimuli analogous to COX-2 and has been shown to be the major producer of PGE_2 (199-201).

Transcriptional Regulation of Microsomal PGES-1

It is now widely accepted that COX-2 and mPGES-1 expression are functionally coupled (193,200,202,203). Early studies of the COX-2 gene identified a number of transcription factor binding sites such as NF-kappa B, CRE, E-box and NF-IL6 that are required for its inducible expression (155,157,160,204). The gene encoding mPGES-1 is located on the long arm of chromosome 9, 9q34.3. The genome consists of two introns and three exons spanning ~14.8 kb; the promoter region is GC-rich and lacks a TATA box. In 2000, work conducted by Forsberg et al. (205) provided insight into the structure and potential regulation of mPGES-1. Their functional analysis of a 0.6 kb mPGES-1 promoter fragment illustrated a strong increase in promoter activity following IL-1β treatment. Later studies by Naraba et al. (206) and Moon et al. (207) revealed that the transcription factor, Egr-1 was capable of binding to a region within the proximal promoter of mPGES-1 and was important for its gene transcription. More recently, a regulatory region for NF-kappa B was identified within the promoter region of mPGES-1 and mutational analysis revealed that this region was important for mPGES-1 promoter activation (208,209). In Chapter 3, a more detailed overview of mPGES-1 gene regulation will be provided.

Physiological Relevance of Microsomal PGES-1 Gene Expression Evaluated in Knockout Mice

Much of what is known about mPGES-1 activity has been centered on COX-2 and PGE_2 expression. Recently, the generation of mPGES-1 null mice has revealed a number of interesting details as to its function and most importantly its physiological significance. Overall, the

phenotype of the null mice appears to be no different than that of the wild-type mice; they develop normally and are capable of reproduction. Studies focusing on endotoxin-induced shock (210), arthritis (211), fever (210), pain perception/blood pressure (212), stroke and anorexia have utilized mPGES-1 null mice (213,214). In an early study by Levin et al. (215), they showed that the administration of exogenous PGE_2 to the ventricular system of the brain and not IL-1β suppressed food intake in mPGES-1 -/- animals. A later study by Pecchi et al. (216) also confirmed that administration of PGE_2 induces anorexia by suppressing food intake in mPGES-1 -/- mice whereas IL-1β did not decrease food intake in these mice. In studies analyzing pain hypersensitivity (211,217,218), researchers found that mPGES-1 -/- animals exhibited a lower response to stimuli compared to wild-type animals and interestingly, the prostanoid profile was altered in these animals. In both collagen-induced arthritis and collagen antibody-induced arthritis models, both the wild-type and mPGES-1 -/- animals developed arthritis but the degree of severity was 50% less in the knockout animals compared to the wild-type animals (211,218). Intuitively, targeted disruption of the mPGES-1 gene leads not only to a reduction of its enzymatic activity but also to an overall reduction in PGE_2 production, strongly implicating this synthase in the regulated production of PGE_2 (35,219).

Expression of Microsomal PGES-1 in Cancers

Like COX-2 and PGE_2, mPGES-1 is highly expressed in many cancers including breast, colon, ovarian and lung (220-224). Yoshimatsu et al. (199,225) evaluated mPGES-1 expression in both lung cancer and colorectal adenomas. Their studies revealed that in over 80% of colorectal tumors mPGES-1 was expressed. The authors also analyzed the effect of the cancer-causing gene, *Ras*, on mPGES-1 expression and found that *Ras* expression led to a marked increase in mPGES-1 promoter activity. They analyzed COX-2 expression in the tumors and found that COX-2 was also induced about 80% and a known inducer of COX-2 expression,

TNF-α, stimulated an up-regulation of both mPGES-1 and COX-2 expression (199). In their subsequent paper, the authors evaluated mPGES-1 expression in non-small cell lung cancer harboring oncogenic *Ras* and nontransformed cells. They found that mPGES-1 and COX-2 expression were up-regulated in transformed cell lines harboring the mutant *Ras* gene, and both mPGES-1 and COX-2 expression were up-regulated in response to treatment with TNF-α (225). It should be noted that in both of these studies the molecular mechanism involved in the regulation of mPGES-1 was not addressed. Finally in 2004, Chang et al. (226) demonstrated that PGE_2 induced tumor-associated angiogenesis and treatment with the NSAID, indomethacin, inhibited tumorigenesis and tumor-associated angiogenesis in murine mammary glands.

In conclusion, there are many studies which support the role of PGE_2 in tumorigenesis. These studies also implicate the role of COX-2 in the formation of PGE_2 and the recent discovery of mPGES-1 and its up-regulation in many cancers has been shown to correlate with COX-2 expression. Epidemiological studies have evaluated the role of NSAIDs and COXIBs in many cancers and disease states, and although they have been shown to reduce the risk of tumor progression and the inflammatory response, they are associated with devastating side effects including increased risk of gastrointestinal complications, such as bleeding ulcers and adverse cardiovascular effects. Therefore, mPGES-1 represents a new and potentially advantageous therapeutic target for the development of drugs aimed at suppressing PGE_2 production without affecting the general prostanoid profile and potentially without major side effects.

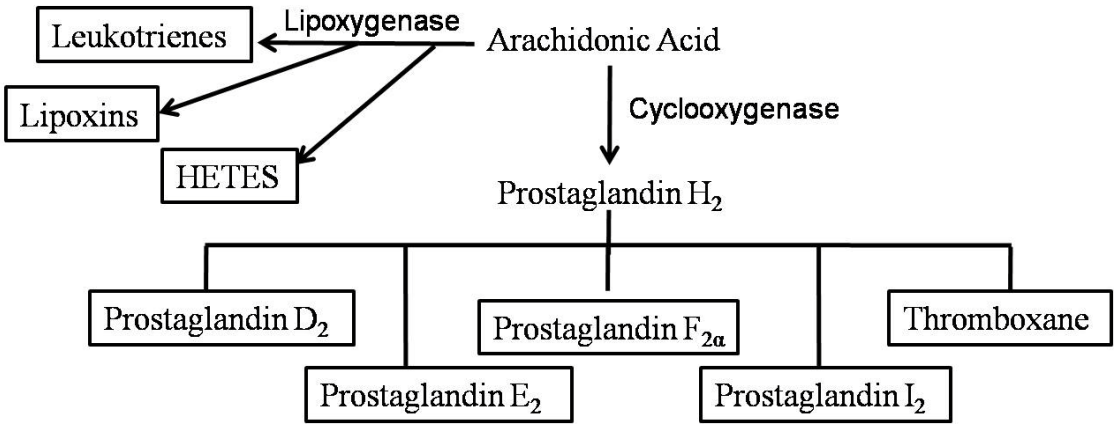

Figure 1-1. Arachidonic Acid Pathway. This diagram illustrates two different pathways involved in arachidonic acid metabolism. The first pathway is mediated by cyclooxygenase, which converts arachidonic acid to a central intermediate prostaglandin H_2. Prostaglandin H_2 can be further metabolized to yield, PGD_2, PGE_2, $PGF_2\alpha$, PGI_2 and thromboxane. In the second pathway, lipoxygenase converts arachidonic acid to leukotrienes, lipoxins or hyroxyeicosatetraenoic acid (HETE).

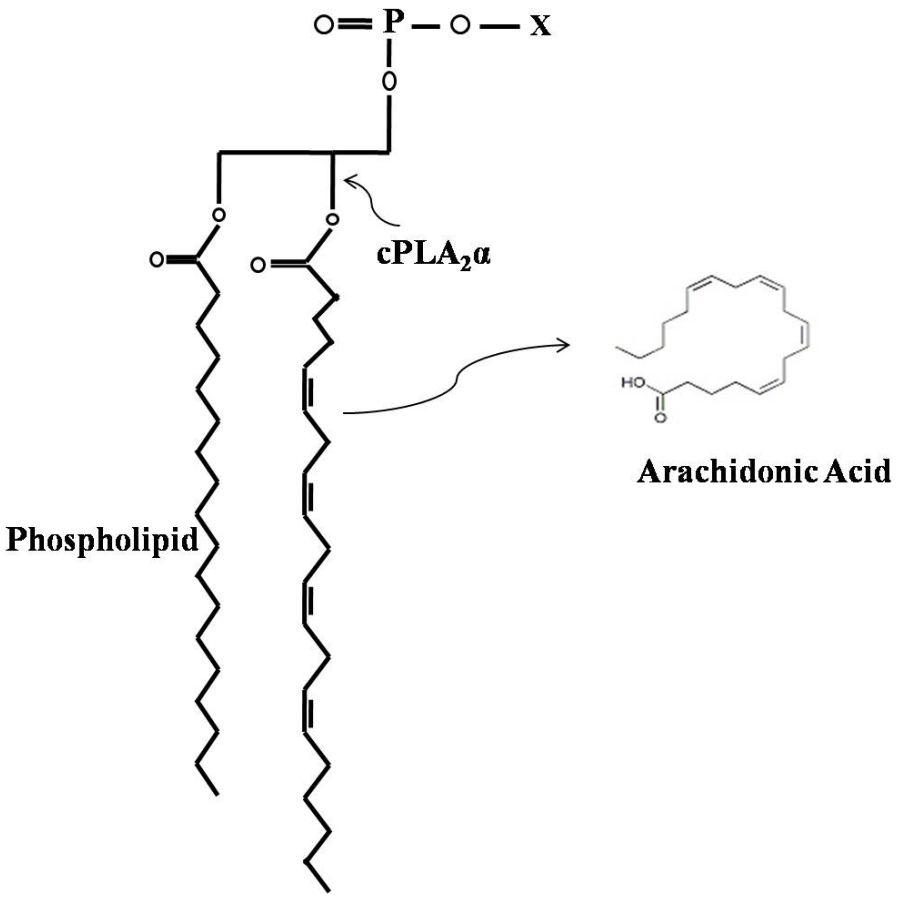

Figure 1-2. Cleavage of arachidonic acid from membrane phospholipids. The diagram depicts the liberation of arachidonic acid from phospholipids. $cPLA_2\alpha$ preferentially cleaves membrane phospholipids at the sn-2 position, liberating free arachidonic acid which is further metabolized by downstream enzymes.

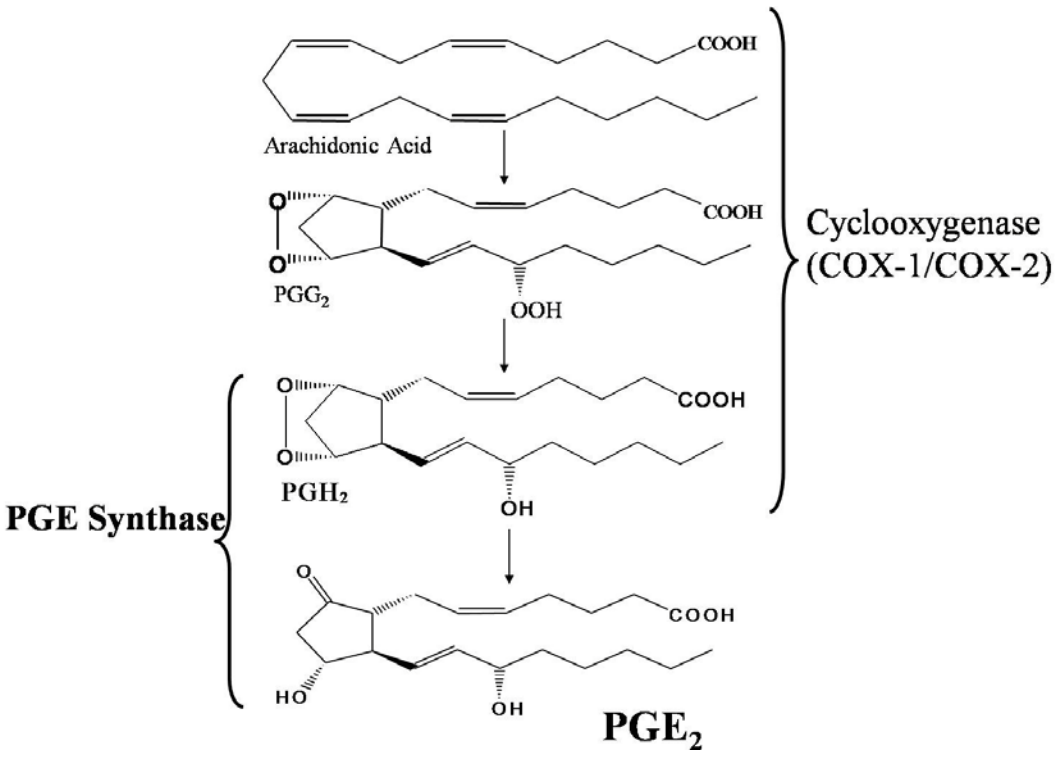

Figure 1-3. Synthesis of PGE_2. Free arachidonic acid is metabolized by the action of cyclooxygenase 1 and 2 in a series of redox reactions to PGH_2. PGH_2 is then converted to PGE_2 in a reaction catalyzed by the PGE_2-specific synthases.

CHAPTER 2
MATERIALS AND METHODS

Materials

FUGENE® 6 transfection reagent (11988387001), interleukin-1β (IL-1β) (201-LB) and complete protease inhibitor cocktail (11697498001) were purchased from Roche Applied Science (Indianapolis, IN). Restriction endonucleases, T4 DNA Ligase (M0202L), Vent Polymerase (M0254L), Taq Polymerase (M0267L) and Klenow (large fragment of *E. coli* DNA Polymerase) (M0210L) were purchased from New England Biolabs (Boston, MA). $AACOCF_3$ (100109), pyrrolidine (525143), Bay-11-7082 (196870), ALLN (208719), SP600125 (420119), PD98059 (513000), SB203580 (559389), SB202190 (559388) and L-α-lyso-lecithin (440154) were purchased from Calbiochem (Gibbstown, NJ). DNase I (LS006342) was purchased from Worthington Biochemical (Lakewood, NJ). Ham's F12K media (N3520), PD146176 (P4620), curcumin (C1386), actinomycin D (A9415) and proteinase K (P6556) were purchased from Sigma-Aldrich (St. Louis, MO). Ciglitazone (71730), Luteolin (10004161), MK886 (10133), NDGA (70300), indomethacin (70270), mPGES-1 monoclonal and polyclonal antibodies (10004350, 160140) were purchased from Cayman Chemical (Ann Arbor, MI). Dulbecco's modified eagle medium (DMEM 10-013-CV) was purchased from Mediatech Inc. (Manassas, VA). Phospho-$cPLA_2$ (ser505) (2831), phospho-MSK1 (Ser376) (9591) and phospho-MKK3/MKK6 (Ser189/207) (9231) antibodies were purchased from Cell Signaling Technologies (Dover, MA). Protein AG agarose beads (SC-2003), anti-His antibody (SC-803), Egr-1 antibodies (SC-189, SC-101033) and C/EBPβ antibodies (SC-150, SC-7962) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Hyperfilm MP (28906846) and ECL™ western blotting system (RPN2108) were purchased from GE Healthcare (Piscataway, NJ) and Bicinchoninic acid protein assay kit from Pierce (Rockland, IL). A

Quikchange site-directed mutagenesis kit with XL-1 Blue competent cells (200518-5) was purchased from Stratagene (La Jolla, CA). QIAquick Nucleotide Removal Kit 28306), Qiagen Plasmid Maxi Kit (12163), Hispeed Plasmid Midi Kit (12643), QIAquick Gel Extraction Kit 28706), QIAquick PCR purification kit (28106), RNeasy Mini Kit (74106), QIAprep Spin Miniprep Kit (27106), RNase Free DNase Kit (79254) and the siRNA for Luciferase (SI03650353) were purchased from Qiagen (Valencia, CA). iTaq™ SYBR® Green Supermix with ROX (172-5851), Criterion precast Tris-HCl gels (10% and 15%) (345-0009, 345-0019) and Zeta-Probe nitrocellulose membrane (162-0115) were purchased from Bio Rad (Hercules, CA). A random-primer DNA labeling kit (18187-013), TOPO® XL PCR Cloning Kit with One Shot Chemically Competent Cells (K4700-10) and SuperScript™ first strand synthesis kit (11904-018) were purchased from Invitrogen Technologies (Carlsbad, CA). The siRNAs for rat C/EBPβ (L-092218-00), human C/EBPβ (L-006423-00), human Alox15B (L-009026-00), cyclophilin B (D-001136-01) and DharmaFECT 1 transfection reagent (T-2001-02) were purchased from Dharmacon, Inc (Lafayette, CO).

Methods

Cell Culture

Human lung fibroblast, HFL-1 cells (ATCC CCL 153) and a rat pulmonary epithelial-like cell line, L2 (ATCC CCL 149) obtained from ATCC were maintained in continuous cell culture in Ham's F12K media supplemented with 4 mM glutamine, ABAM (0.1 mg/mL streptomycin, 0.25 μg/mL amphotericin B and 10 μg/mL penicillin G) and 10% FBS at 37°C and 5% CO_2. Mouse embryonic fibroblasts (MEF): wild type and C/EBPβ -/- cells were provided by Dr. P. Johnson, NIH via Dr. Michael Kilberg; wild type and p38α -/- cells were provided by Dr. A. Nebreda, EMBL; wild type and p38β -/- cells were provided by Dr. A. Choi, Harvard Medical School and wild type and MKK3/6 -/- cells were provided by Dr. R. Davis University of

Massachusetts. All MEF cell lines were maintained in DMEM media supplemented with 10% fetal bovine serum and ABAM (10 µg/mL penicillin G, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B) in continuous culture at 37°C in humidified air with 5% CO_2. For plasmid transfections and protein over-expression, cells were seeded on 10 cm or 150 mm dishes. For cytokine treatment, cells were seeded on 10 cm dishes and for inhibitor studies cells were seeded on 10 cm dishes then treated with pharmacologic inhibitors for 1 h prior to exposure with IL-1β. For siRNA transfections cells were seeded on 35 mm dishes.

Plasmids, Probes and Site-Directed Mutagenesis

An MluI site was introduced into a pUC12-based human growth hormone (hGH) expression vector using site-directed mutagenesis by Dr. JD Herlihy in our lab. The hGH reporter constructs were generated by sub-cloning the following mPGES-1 promoter fragments, -1104/+160 (-1.1 kb) and -434/+160 (-0.6 kb) into the promoter-less, hGH expression vector using the Hind III and BamHI sites, all numbering relative to the start of transcription (+1) of the mPGES-1 gene. An Egr-1 consensus site and a C/EBPβ consensus site in the mPGES-1 promoter construct, identified by TESS – transcription element search software (227) were deleted by site-directed mutagenesis. Briefly, 10 ng of -1.1 kb promoter construct was used as the template and 125 ng of each mutagenesis primer were added to 1 µL dNTP mixture, 1.5 µL dimethyl sulfoxide (DMSO), 5 µL 10X reaction buffer, 1 µL Pfu Turbo Polymerase and mixed together, then brought to a final volume of 50 µL with sterile double distilled water. The reaction was conducted in a PTC 100 peltier thermal cycler using the following parameters: Cycle 1 (95 ºC, 30 sec) 1x, Cycle 2 (95 ºC for 45 sec, 60 ºC for 1 min, 68 ºC 7 min) 18x. The reaction was stopped by incubation on ice for 5 min and 1 µL DpnI was added to digest the methylated parental strand, leaving the mutated product, which is then transformed into XL-1-Blue competent cells and incubated on agar containing ampicillin. Resultant colonies containing

the recombinant plasmids were isolated and sequenced for verification of the mutation. The following mPGES-1 genomic fragments were sub-cloned into pGH1.1 at the MluI site: -10.7/-6.4, -10.7/-9.6, -10.1/-9.0, -9.5/-8.5, -8.6/-6.4, -7.6/-6.4, -8.6/-8.1 and -8.1/-7.6, all numbering relative to the start of transcription (+1) of the mPGES-1 gene. The primer sequences are listed in Table 2-1. The -10.7/-6.4 fragment was also cloned into an hGH expression vector containing the heterologous viral thymidine kinase promoter at the NdeI site of this vector. The three potential C/EBPβ consensus sites in the -8.6/-8.1 fragment were also identified by TESS and deleted by site-directed mutagenesis, using the primers listed in Table 2-1. The mPGES-1 probe used for northern blot analysis were amplified from the cDNA sequence using the forward primer 5'-GAATTCGCCAGAGATGCCTGCCCACA-3' and reverse primer 5'-GAATTCACACACGGGCACACACACAGGC-3'. The 0.7 kb growth hormone probe was generated by restriction digest of the growth hormone cDNA using the XbaI and HindIII sites.

Transient Transfection

Prior to transfection and treatment with cytokines, HFL-1 cells were cultured as previously described and transfected at approximately 60 – 70% confluency. 5 µg of the indicated plasmid was transfected into HFL-1 cells using the Fugene 6-Reagent protocol from Roche. Briefly, in a 1.5 mL tube, 5 µg of DNA was complexed with 15 µg of Fugene 6-Reagent (ratio of 1:3 DNA to Fugene 6-Reagent) in 580 µL serum free media and the complex was incubated at room temperature for 20 min, during this time the cells were washed 1x with 1X PBS and the media replaced. The DNA-complex was added to the cells and incubated at 37°C for 3h. The cells were again rinsed 1x with 1X PBS, media replaced and then incubated overnight at 37°C in humidified air with 5% CO_2. At 24 h post-transfection, each 10 cm plate of cells was trypsinized and split into two 10 cm plates and incubated overnight. This batch transfection method controls for equal transfection efficiency for each transfected construct. Forty hours post-transfection,

cells were stimulated with or without 2 ng/mL of IL-1β for 8 h. As a control a promoter-less construct was also transfected to ensure that the transfection or the hGH plasmid does not have any effect on the mPGES-1 message.

RNA Isolation, Northern Blot and Hybridization

Total cellular RNA was isolated as described by Chomczynski and Sacchi with modifications (96,228) or using the Qiagen RNeasy Kit. After treatment with cytokines, cells were rinsed 1x with 1X PBS, lysed on the plate by the addition of 500 μL GTC solution (4M guanidinium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% sarcosyl and 0.1M β-mercaptoethanol) and then lysate transferred to a 1.5 mL tube. The mixture was then vortexed briefly to aid cellular lysis, then 50 μL 2M sodium acetate pH4.0 followed by 500 μL water-saturated phenol was added to the tube. The lysate was then inverted 5x to mix and incubated at room temperature for 5 minutes. Next, 110 μL of 49:1 chloroform:isoamyl alcohol mixture was added to the lysate and the tube was shaken to mix and centrifuged at 13200 rpm for 15 minutes. The aqueous phase was removed to a fresh 1.5 mL tube and an equal amount of isopropanol was added to the sample which was then incubated at -20°C for one hour. The sample was centrifuged at 13,200 rpm for 30 minutes at 4°C and the pellet was re-suspended in 75 μL pyrocarbonate (DEPC) treated double distilled water. The RNA was precipitated by the addition of 2 M lithium chloride followed by incubation -20°C for 30 minutes. After centrifugation at 13,200 rpm for 30 minutes 4°C, the RNA pellet was washed with ethanol, briefly dried and re-suspended in 50-150 μL DEPC-water. For Qiagen RNeasy Kit extraction protocol, after treatment with cytokines, cells were rinsed 1x with 1X PBS, then lysed on the plate by the addition of 600 μL Buffer RLT and the lysate transferred to a 1.5 mL tube. The tube was then vortexed briefly to aid cellular lysis, an equal volume of 70% ethanol was added to precipitate the RNA and mixed by pipetting up and down. The RNA was bound by passing the mixture

over an RNeasy spin column and spinning the column at 13,200 rpm for 15 seconds. The flow-through was discarded and 350 μL of Buffer RW1 was added to the column. The column was again spun at 13,200 rpm for 15 seconds, the flow-through was discarded and 80 μL DNase solution (10 μL DNase 1, 70 μL Buffer RDD) was added to the column. After a 15 minute incubation at room temperature the reaction was stopped by the addition of 350 μL Buffer RW1. The column was rinsed 2x with 500 μL of Buffer RPE spinning at 13,200 rpm for 15 seconds and 2 minutes, respectively. The RNA was eluted from the column by adding 50 μL DEPC-water, incubation at room temperature 2 minutes then spinning at 13,200 rpm for 1 minute. The concentration was determined by spectrophotometrical analysis at A_{260}. For northern blot analysis, 20 μg of total RNA was size-fractionated on a 1% agarose-formaldehyde gel, running at 40V overnight in 1X TBE (89 mM Tris, 89 mM Boric Acid and 2 mM EDTA). The size fractionated RNA was electro-transferred to a nylon membrane and UV cross-linked for 2 minutes. The membrane was incubated for one hour in a prehybridization buffer (0.45M sodium phosphate, 6% sodium dodecyl sulfate (SDS), 1mM EDTA and 1% bovine serum albumin (BSA). A random primed double stranded ^{32}P-labeled gene-specific probe for (human mPGES-1, hGH or human large subunit ribosomal L7a) was added to the prehybridization solution and the membrane incubated overnight (mPGES-1 at 65°C, hGH or L7a at 61°C). The membrane was washed three times for 10 minutes at 60-65°C in a high stringency buffer (0.04M sodium phosphate, 2mM EDTA and 1% SDS) and exposed to X-ray film.

Transcription Rate Determination

Total RNA was isolated from HFL-1 cells at the indicated time points after treatment with IL-1β and DNase treated to eliminate genomic DNA contamination. To measure the transcription rate for mPGES-1, primers specific for Intron 2 and Exon 3 were used for real-time

RT-PCR after first strand cDNA synthesis to measure the level of pre-mRNA or heterogeneous RNA (hnRNA). The primers used for hnRNA amplification were sense primer 5'-TGGCTGTGAATGGATTTGAGTG-3' and antisense primer 5'-AGGAAAAGGAAGGGGTAGATGG-3'. This method is based on the published work of Lipson and Baserga (229). To rule out any amplification from contaminating genomic DNA, an equal amount of RNA following first strand cDNA synthesis without the addition of Superscript™ II reverse transcriptase was used as a negative control.

First-Strand DNA Synthesis and Real-Time RT-PCR

One (1 μg) microgram of total RNA was used to generate first strand cDNA for real-time PCR analysis using a SuperScript™ first strand synthesis kit. First, in a 0.5 mL PCR tube, 1 μg of total RNA was mixed with 10 mM dNTPs, 0.5 μg oligo dT and DEPC-water to a final volume of 10 μL and denatured by incubation at 65°C for 5 minutes then 4°C. Next, 9 μL of reaction mixture (10X RT Buffer, 25 mM DEPC-$MgCl_2$, 0.1 mM DTT, 40 U/μL RNaseOUT™ Recombinant RNase Inhibitor) was added to the tube which was then incubated at 42°C for 2 minutes. 1 μL (50 U) of Superscript™ II RT was added to the tube and the reaction was further incubated at 42°C for 50 minutes. The reaction was terminated by incubation at 70°C for 15 minutes and the tubes were spun briefly in a microcentrifuge. To remove template RNA, 1 μL (40 U) of RNase H was added to the tube and the reaction incubated at 37°C for 20 minutes. The tubes were spun briefly and sterile double-distilled water was added to a final volume of 100 μL. Real-time PCR was conducted using 2 μL cDNA as the template, 0.3 μM of each primer, 12.5 μL of iTaq SYBR® Green Supermix with ROX and water to a final volume of 25 μL. The primers used for amplification are as follows: human mPGES-1 sense primer, 5'-GCCGCCGTGGCTATACC-3', and antisense primer, 5'-GGTTCCCATCAGCCACTTC-3',

hGH, sense primer 5'-GAACCCCCAGACCTCCCT-3', and antisense primer 5'-CATCTTCCAGCCTCCCCAT-3', mouse mPGES-1 sense primer, 5'-TTAGAGGTGGGCAGGTCAGAG-3', and antisense primer, 5'-CCACTCGGGCTAAGTGAGAC-3', rat mPGES-1 sense primer 5'-CGCAACGACATGGAGACGA-3', and antisense primer, 5'-GCGTGGGTTCATTTTGCC-3', human cPLA$_2\alpha$ sense primer 5'-CGTGATGTGCCTGTGGTAGC-3', and antisense primer, 5'-TCTGGAAAATCAGGGTGAGAATAC-3'. Each real-time PCR reaction was conducted using the Applied Biosystems 7000 sequence detection system (Foster City, CA) with the following parameters: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. At completion, the melting curves were acquired by a stepwise increase of the temperature from 55°C to 95°C to ensure that a single product was amplified in the reaction. Cyclophilin A levels were also measured concurrently as the internal control utilizing the following primers: human cyclophilin A sense primer, 5'-CATCCTAAAGCATACGGGTCC-3' and antisense primer, 5'-GCTGGTCTTGCCATTCCTG-3', mouse cyclophilin A sense primer, 5'-GCGGCAGGTCCATCTACG-3', and antisense primer, 5'-GCCATCCAGCCATTCCAGTCT-3', rat cyclophilin A sense primer, 5'-GGTGGCAAGTCCATCTACGG-3', and antisense primer, 5'-TCACCTTCCCAAAGACCACAT-3'. Each PCR reaction was done in triplicate based on samples from three independent experiments and the ΔΔCT method was used to determine the relative fold expression, normalized to cyclophilin A as described by Livak et al. (230).

Immunoprecipitation Assay

HFL-1 cells were grown as described and treated with 2 ng/mL of IL-1β as indicated. Total cell extracts were prepared in TNE lysis buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% NP-40, 10 µg aprotinin, 10 µg leupeptin, 10 µg pepstatin, 5 µL of 200

mM PMSF, 25 µL DTT) and incubated at 4°C overnight with the monoclonal antibody against the indicated protein. Protein AG agarose beads were washed 4x in TNE lysis buffer then incubated with the lysates at 4°C for 2 h. Bead complexes were washed 4x with TNE lysis buffer and proteins were eluted using 30 µL of 1X Laemeli buffer followed by immunoblot analysis.

Protein Isolation

For immunoblot analysis, protein lysates were prepared from HFL-1 as follows; on ice cells were washed twice with cold 1X PBS and lysed by the addition of 50 ul of Tris lysis buffer (1M Tris-HCl pH7.5, 5M NaCl, 0.5M EDTA pH8.0, Triton X-100 plus 1X protease inhibitors). The cell membrane was further disrupted by the use of a hand held homogenizer and incubated on ice for 10 min. The lysates were centrifuged at 14,000x g for 15 min at 4 ºC to remove cellular debris. The supernatant was removed to a fresh pre-chilled 1.5 mL tube and the protein concentration was determined by the bicinchoninic acid (BCA) assay in triplicate.

Immunoblot Analysis

Total cell extracts or imunoprecipitates were separated on a 10% or 15% Tris-HCl polyacrylamide gel, respectively and electro-transferred to a nitrocellulose membrane. The membrane was blocked overnight at 4 °C with 7.5% non-fat dry milk in TBST (10 mM Tris-HCl, pH 7.5, 0.1% (v/v) Tween 20, 200 mM NaCl). The indicated primary antibody (Egr-1 1:200, C/EBPβ 1:600, mPGES-1 1:400, Alox15B 1:1000) was added to the membrane which was then incubated at 4°C overnight. The membrane was washed 3x with TBST, incubated with a peroxidase-conjugated secondary antibody (rabbit 1:10,000) for 1 h, washed again 3x with TBST and subjected to chemiluminescent detection. The membrane was soaked in the detection agent (equal mixture of ECL Advance Solution A and B) for 1 minute then exposed to autoradiography film.

DNase I Hypersensitive Site Analysis

HFL-1 cells were incubated in the presence or absence of 2 ng/mL IL-1β for 8 h, rinsed 1X with PBS then trypsinized for 10 min at 37ºC. The cells were resuspended in 4 mL of permeabilization buffer (150 mM Sucrose, 80 mM KCl, 35 mM HEPES pH 7.4, 5 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$) containing 0.1% L-α-lyso-lecithin on ice for ~2.5 min. The reaction was stopped by the addition of 40 mL permeabilization buffer and the cells pelleted for 5 minutes at 4ºC. The permeabilized cell pellets were resuspended in 3.2 mL of permeabilization buffer. 300 µL of permeabilized cell suspension was digested with increasing concentrations of DNase I for 4 minutes at 37ºC. The reactions were terminated by the addition of DNA lysis buffer (4% SDS, 0.2 M EDTA and 800 µg/mL proteinase K). Genomic DNA was purified by incubation at 50ºC for 3 h followed by organic extractions and precipitation with ethanol. Samples were then resuspended in 100-200 µL of TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). The samples were then digested with the restriction enzyme, HindIII, in a total volume of 300 µL. The digests were size-fractionated on a 0.8% HGT agarose gel in TAE buffer, pH 7.8 (40 mM Tris, 3 mM NaOAc, 1 mM EDTA, 4 mM NaOH) overnight at 40 V to resolve the DNA fragments. The gel was then alkaline-denatured by incubation in 50 mM NaOH for 30 minutes then 0.1M TBE (1M TBE: Tris 242g, Boric Acid 107g, EDTA 6g) 2x for 30 minutes. The gel was electro-transferred to a nylon membrane, and cross-linked to the membrane with UV light 2 minutes. The membrane was hybridized with an end-specific single copy DNA probe at 61 ºC. The DNA probes used were generated by PCR from the human mPGES-1 genomic clone using the following primers; 19.6 kb end forward 5'-GCTTAATGCATGAAGTGGTTAC-3', reverse 5'-AAGATGAAGCTGCCTTTGAG-3' and 6.8 kb end forward 5'-TCAGGATGCAGAGCCAAGC-3', reverse 5'-CCAGTGAACTACAGGCACCAG-3'. The

DNA probe was radiolabeled using the random primer DNA labeling system and hybridized to the membrane. Hybridization and autoradiography were performed as previously described.

Chromatin Immunoprecipitation Analysis

Chromatin immunoprecipitation (ChIP) analysis was performed according to a modified protocol from Upstate Biotechnology, Inc. (Charlottesville, VA). HFL-1 cells were grown to 90% confluency on 150 mm plates and cross-linked with 1% formaldehyde for 10 min at room temperature and quenched by the addition of 125 mM glycine for 5 min. The cells were then scraped into 50 mL conical tubes and centrifuged at 3000 rpm for 15 min at 4 ºC. The pellet was washed 2x with 1X PBS and resuspended in cold swelling buffer (5 mM PIPES pH 8.0, 0.5% NP-40, 85 mM KCl plus 1X protease inhibitors) and incubated on ice for 10 min. The swelled cells were then centrifuged at 5,000 rpm for 5 min at 4 ºC and the cell pellet was gently resuspended in 1 mL lysis buffer (1% SDS, 50 mM Tris pH 8.1, 10 mM EDTA and 1X protease inhibitors). The lysates were sonicated to ~500bp fragments using a Branson Model 500 dismembrator (Fisher Scientific) at 40% amplitude for 5x 30 sec bursts with 2 min rest on ice between bursts. The sonicated samples were removed to 1.5 mL tubes and centrifuged at 13,000 rpm for 5 min at 4 ºC to clear cell debris. The supernatants were diluted 1:10 in ChIP dilution buffer (0.1% SDS, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl and 1.1% Triton X-100), then pre-cleared with 500 µL of Protein A sepharose beads for 2 h at 4 ºC. The pre-cleared supernatants were then split into 1 mL aliquots, and 2 µg of indicated antibodies were added and tubes were incubated overnight at 4 ºC. The next day, 60 µL protein A or G sepharose beads blocked with 30% BSA were added to each tube to capture the complex. Following incubation at 4 ºC for 2 h, the complexes were isolated by centrifugation at 1,000 rpm for 2 min, 500 µL of IgG control samples were removed for Input controls and the complexes were washed as follows: once with low salt (0.1% SDS, 1% Triton X-100 (v/v), 20 mM Tris pH 8.1, 2 mM

EDTA, 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100 (v/v), 20 mM Tris pH 8.1, 2 mM EDTA, 500 mM NaCl), LiCl (250 mM LiCl, 1% NP-40, 1% sodium deoxycholate (DOC), 10 mM Tris pH 8.1, 1 mM EDTA) and three times with TE (10 mM Tris pH 8.0 and 1 mM EDTA pH 8.0). The samples were then eluted with 500 μL of elution buffer (1% SDS and 100 mM $NaHCO_3$) with incubation at 37 ºC and rocking for 30 min. The eluted samples were centrifuged at 2,000 rpm for 2 min at room temperature, and the supernatants were removed to a fresh 1.5 mL tube. To remove contaminating protein, the eluted samples and Input controls were treated with the addition of the following solutions to reach final concentrations of 11 mM EDTA, 200 mM NaCl, 44 mM Tris pH 7.0, then 2 μL of proteinase K (20 mg/mL) and incubation at 45 ºC for 1 h followed by reverse cross-link at 65 ºC for 4 h. The samples were purified with the Qiagen PCR kit and subjected to real-time RT-PCR analysis. The forward primer 5'-ACAGCTCTGGGCGCACAC-3' and reverse primer 5'-TGGGGAAATGGGAATGACTG-3' were used to amplify region -8.6 to -8.1 kb; the forward primer 5'-CGGCAACTGCTTGTCTTTCTC-3' and reverse primer 5'-TCTTGATGACCAGCAGCGTG-3' were used to amplify the promoter region of human mPGES-1. The forward primer 5'-GCATCAAAAACATCACTCCCTCT-3' and reverse primer 5'-ACTCCAGCTTGGGCAACAGA-3' were used to amplify the 3'UTR and the forward primer 5'-AGAAGCGTAAACATCACTCTCCTC-3' and reverse primer 5'-ACAGCCTCACAGACATACCCAG-3' were used to amplify the 5'UTR of mPGES-1 as negative controls. All results are expressed as a fraction of the total isolated chromosomal DNA (input) prior to immunoprecipitation or relative to IgG, as specified.

Short Interfering RNA (siRNA) Analysis

HFL-1 cells were seeded on 35 mm plates at 50% confluency and transfected with a final concentration of 100 nM *SMART*pool® C/EBPβ siRNA, Alox15B siRNA or a cyclophilin-

specific siRNA (Dharmacon) using 5 μL of Dharma*FECT*™ 1 siRNA transfection reagent (Dharmacon) according to manufacturer's protocol. Briefly, using 15 mL tubes, in tube A, 100 μM of siRNA was diluted in 1X siRNA buffer and mixed with an equal volume of serum free media. In tube B, 5 μL of the reagent diluted in serum free media to a final volume equal to that of tube A. Both tubes were incubated at room temperature for 5 min, then the contents of both tubes were combined and the single tube was incubated at room temperature for 20 min. Depending on the number of plates used, complete media was then added to the tube and the contents divided equally among the plates. Treatment with Dharma*FECT*™ 1 without siRNA was used to control for transfection reagent specific effects. After 72 h incubation, one set of plates were treated with 2 ng/mL of IL-1β for 4 h. Protein and total RNA were isolated and analyzed by immunoblot analysis or reverse transcription followed by real-time RT-PCR.

Densitometry and Statistical Analysis

All densitometry was quantified from autoradiography films using a Microtek scan maker 9600XL and analyzed with NIH Scion Image analysis software. The relative fold-induction was determined for the mPGES-1 mRNA band or the hGH mRNA band, normalized to the L7a ribosomal protein internal control. For real-time RT-PCR analysis, each reaction was done in triplicate and the ΔΔCT method was used to determine the relative fold expression, normalized to cyclophilin A. Data points are the means from at least three independent experiments and the error bars represent the standard error of the means (SEM). An asterisk (*) denotes significance as determined by a Student's t-test to a p value ≤ 0.05 and (**) denotes a p value ≤ 0.01.

Table 2-1. Primers used for generating mPGES-1 fragments

Primer Pairs	Primer Sequence 5' to 3
Human mPGES-1 Promoter -434/+160	F -AAGCTTTCCATTGTCCAGGCTGAGTGT R -GGATCCTTCTTCCGCAGCCTCACTTG
Human mPGES-1 Promoter -1.1/+160	F -AAGCTTAGAGTCAGTTGATAGGTCTTTCGGG R -GGATCCTTCTTCCGCAGCCTCACTTG
Human mPGES HS2 Fragment	F -ACGCGTCCGGCAGTCTGAGCTGAGT R -ACGCGTTGGCCCTGGGTCCTGACT
Human mPGES-1 (-10.7 to -9.6)	F -ACGCGTCCGGCAGTCTGAGCTGAGT R -ACGCGTGTCATCACGCCTGACGGAC
Human mPGES-1 (-10.1 to -9.0)	F -ACGCGTCTAAAGGGTGTCTGGCCATTAGG R -ACGCGTCCACGGGCTGCAGAGGAG
Human mPGES-1 (-9.5 to -8.5)	F -ACGCGTGTCAGGAGTTCAAGACCAGCC R -ACGCGTTGGAATTGCACACTTGAAGATG
Human mPGES-1 (-8.6 to -6.4)	F -CCGTCAGGGACGCGT/CCCTGCATTTAACGC R -GCGTTAAATGCAGGG/ACGCGTCCCTGACGG
Human mPGES-1 (-8.6 to -8.1)	F -ACGCGTAGAAGGAGAGGGCGGCATC R -ACGCGTGGAGAGTTGCCCAGGCTAGAGT
Human mPGES-1 (-8.1 to -7.5)	F -ACGCGTCTGGGCAACTCTCCGTCTCA R -ACGCGTGCAGTGAGCCATGCTGTGATC
Human mPGES-1 (-7.6 to -6.4)	F -ACGCGTTGCTTCCGGCCTGTTTATTT R -ACGCGTTGGCCCTGGGTCCTGACT
C/EPBβ Site 1 (-8.6 to -8.1) Δ1	F -GTTCAGGCCGTCTGT/TATTTACCAAGCACAGCTC R -GAGCTGTGCTTGGTAAATA/ACAGACGGCCTGAAC
C/EPBβ Site 2 (-8.6 to -8.1)Δ2	F -CATTGGTACAGTCACAATA/ATCTTTACCATCCATTTCC R -GGAAATGGATGGTAAAGAT/TATTGTGACTGTACCAATG
C/EPBβ Site 3 (-8.6 to -8.1)Δ3	F -TTGCAACCATCTTTACCATCC/CATTTTCATCATCCCAG R -CTGGGATGATGAAAATGGGAT/GGTAAAGATGGTTGCAA

CHAPTER 3
IDENTIFICATION OF DNASE I HYPERSENSITIVE SITES INVOLVED IN THE INTERLEUKIN 1 BETA (IL-1β) INDUCTION OF MICROSOMAL PROSTAGLANDIN E SYNTHASE-1 (MPGES-1) GENE EXPRESSION

Introduction

Induction of Microsomal PGES-1 Gene Expression by Pro-Inflammatory Cytokines

PGE_2 is known to be involved in a variety of biological processes including reproduction, gastric mucosal protection, pyresis, vasodilatation, sleep and many disease states (3,7,10,28,231,232). The conversion of PGH_2 to PGE_2 is catalyzed by the action of specific PGE synthases, in particular mPGES-1 but while PGE_2 production and activities have been widely studied in a variety of cell types, little is known about the regulation of mPGES-1. Jakobsson et al. (192) were among the first to show that mPGES-1 gene expression is induced by the pro-inflammatory cytokine, IL-1β. Subsequent studies also revealed that mPGES-1 mRNA expression is also induced by LPS (219,233), TNFα (201,234), growth factors such as TGFβ (235), phorbol esters such as phorbol 12-myristate 13-acetate (236) and by the flavonoid epigallocatechin-3-gallate in a number of cell types (237).

Stimulus-Dependent Activity of the Microsomal PGES-1 Promoter

Forsberg et al. (205) analyzed the transcriptional activity of minimal mPGES-1 promoter fragments in transfected human epithelial cells (A549) derived from a lung adenocarcinoma. They generated two promoter fragments, 0.19 kb and 0.65 kb fragments, and observed that the transcriptional activity of each promoter fragment increased ~2 fold in response to treatment with IL-1β. From this study it was postulated that cis-acting elements involved in basal mPGES-1 gene expression are contained within the proximal 0.19 kb promoter fragment. In a later study by Han et al. (238) using a 0.51 kb mPGES-1 promoter construct in human orbital fibroblasts, they illustrated that mPGES-1 promoter activity is up-regulated following IL-1β stimulation,

validating the previous study by Forsberg et al. (205). Their study also evaluated a 1.8 kb COX-2 promoter fragment and revealed that like mPGES-1, COX-2 promoter activity is also induced following IL-1β treatment. Other studies have shown that mPGES-1 promoter activity is up-regulated in response to treatment by phorbol esters (206,239), thapsigargin (239) and TNF-α (234). Alternatively, it should be noted that stimulus-dependent activation of the mPGES-1 promoter is inhibited by the peroxisome-proliferator activating receptor (PPAR) ligands (240,241), inhibition of histone deacetylase activity (242) and inhibition of protein kinase C (243).

Involvement of the Early Growth Response Factor, Egr-1 in the Regulation of Microsomal PGES-1 Expression

Recently, the transcription factor, Egr-1 was found to be important for mPGES-1 gene expression. In 2000, Forsberg et al. (205) identified the presence of two GC boxes within the mPGES-1 proximal promoter by sequence analysis. Later, Naraba et al. (206) evaluated the importance of these two GC boxes in relation to mPGES-1 gene expression. Based on deletion analysis of the promoter region, they showed that Egr-1 binding induced promoter activity 2.0 – 3.0 fold in the presence of a stimulus. Subsequent deletion of the Egr-1 binding site attenuated mPGES-1 promoter activity. A few years later, Moon et al. (207) validated these findings by inhibiting Egr-1 expression, in A549 cells, using an siRNA against Egr-1. They further showed that inhibition of Egr-1 expression led to a significant decrease in mPGES-1 promoter activity. Combined with further studies by other groups (234,237,240,242), a clear role for Egr-1 activity in regulating the inducible expression of the mPGES-1 promoter has been outlined. While Egr-1 is required for mPGES-1 transcriptional activation, suppression of Egr-1 expression does not completely block induced mPGES-1 expression (237). Moreover, treatment with IL-1β, for example causes an ~8 fold induction of steady state mPGES-1 mRNA levels compared to the

published 2 fold induction observed with minimal proximal promoter fragments. Furthermore, mutagenesis of the Egr-1 binding site does not always completely eliminate this level of induction. Therefore, these findings imply that other transcription factors may be involved in the regulation of mPGES-1 gene expression and thus is the basis for our attempts to identify additional regulatory sequences that are potentially responsible for the IL-1β-dependent regulation.

Results

Induction of Microsomal PGES-1 Messenger RNA and Protein Expression by the Pro-Inflammatory Cytokine, IL-1β in Human Lung Fibroblasts

PGE_2 is known to be cyto-protective in the lung and recent studies have shown that mPGES-1 expression is up-regulated in lung fibroblasts in a stimulus dependent manner (15,205,222), therefore human lung fibroblasts (HFL-1) were used as the cell model for studying the regulation of mPGES-1 gene expression. In the initial studies, a dose response with IL-1β was conducted to determine the effective concentration that produced the largest induction of mPGES-1 gene expression. HFL-1 cells were incubated with increasing concentrations of IL-1β (0.5 – 10 ng/mL) and total RNA isolated. Purified RNA was analyzed by real-time RT-PCR and data was evaluated for the level of induction compared to the untreated control which was normalized to 1. As illustrated in Figure 3-1, 2 ng/mL of IL-1β induced mPGES-1 mRNA expression approximately 8 fold; therefore this concentration will be used for all subsequent treatments.

After continued stimulation with IL-1β over the course of 12 h, mPGES-1 mRNA expression was analyzed by northern blot. The results in Figure 3-2 provide a representative northern analysis, and reveal that endogenous steady-state levels of mPGES-1 mRNA expression is induced in a time dependent manner with an apparent maximal induction by 8 – 12 h. To

quantify this increase in mRNA expression, HFL-1 cells were treated with IL-1β over the course of 8 h, total RNA was isolated and subjected to real-time RT-PCR analysis with mPGES-1 specific primers. The chart in Figure 3-3 illustrates that similar to the northern blot analysis in Figure 3-2, mPGES-1 mRNA levels increased approximately 9 fold in a time dependent manner by 8 h.

To demonstrate that the increase in mRNA levels translates into a logical increase in protein levels, mPGES-1 protein expression was evaluated following IL-1β stimulation in HFL-1 cells. There are two commercially available antibodies used to detect mPGES-1 protein but after unsuccessful attempts to detect mPGES-1 protein expression by standard immunoblot analysis, we devised an immunoprecipitation protocol as described in the Materials and Methods. To quantify mPGES-1 protein expression, total protein from control and stimulated cells was immunoprecipitated with a mouse monoclonal antibody to mPGES-1, then size fractionated by SDS/PAGE followed by immunoblotting with a rabbit polyclonal mPGES-1 antibody. The immunoprecipitation analysis in Figure 3-4 revealed that in control cells, there is no detectable mPGES-1 protein expression but following 72 h of IL-1β stimulation, mPGES-1 protein expression was significantly elevated, thereby demonstrating that IL-1β treatment caused a significant induction of both mPGES-1 mRNA and protein levels.

Determination of Microsomal PGES-1 Messenger RNA Decay After Stimulus Removal

The classical experiment to evaluate mRNA half-life involves stimulating cells for a short time period, followed by the addition of actinomycin D to globally inhibit transcription; samples are then analyzed at various time points post treatment to determine the level and time-dependent degradation of the message. Since there is often no detectable basal expression of mPGES-1, the measurement of basal mRNA decay cannot be accurately determined. We therefore chose to evaluate the decay of the induced message by first treating with IL-1β to stimulate induction of

mPGES-1 mRNA followed by removal of the stimulus. HFL-1 cells were stimulated for 8 h with IL-1β, the stimulus was removed and the cells were rinsed 3x with 1X PBS and fresh media was added to the cells. Cells were harvested at specific time points over the course of 12 h, total RNA isolated and analyzed by northern blot. The data in Figure 3-5 reveals that at 6 h post stimulus removal, a rapid decay of the mRNA levels was observed where by 12 h the mRNA levels were almost undetectable.

The IL-1β Induction of Microsomal PGES-1 Gene Expression Requires De Novo Transcription

In order to determine whether the IL-1 induction of mPGES-1 gene expression was a consequence of regulation at the transcriptional level, a global transcriptional inhibitor actinomycin D was utilized and steady state mRNA levels were measured. HFL-1 cells were treated with actinomycin D alone to inhibit global transcription in the absence or presence of IL-1β and mPGES-1 mRNA expression was analyzed by northern blot. The results in Figure 3-6 indicate that treatment with actinomycin D alone did not affect mPGES-1 mRNA expression while actinomycin D did block the IL-1β induction of mPGES-1 mRNA expression.

To directly address whether *de novo* transcription is responsible for the IL-1β-dependent induction of mPGES-1 expression, heterogeneous nuclear RNA levels were evaluated by real-time RT-PCR amplification across an intron-exon boundary. Heterogeneous nuclear RNA (hnRNA) is a pre-mRNA intermediate, that exists prior to splicing, containing both introns and exons. The level of hnRNA present at any given time directly correlates with the presence of *de novo* transcription (229). As an alternative to the classical nuclear run-off assay, the measure of hnRNA is being utilized as an efficient and quantitative assessment of *de novo* transcription. Primers spanning the intron 2/exon 3 boundary were designed and utilized for real-time RT-PCR amplification.

The data in Figure 3-7 illustrates that within 0.5 h of IL-1β treatment, there is a significant increase in hnRNA levels with a maximum at 1 h (~8 – 9 fold) thus indicating that *de novo* transcription is required for the IL-1β induction of mPGES-1 gene expression. The decrease in induction following 1 h is possibly due to the competing rates of new hnRNA synthesis and the time at which intron splicing eliminates the template for the intron specific primer.

Evaluation of the Microsomal PGES-1 Proximal Promoter in the HFL-1 cells

In an attempt to elucidate the mechanism involved in regulating mPGES-1 gene expression, mPGES-1 promoter activation was evaluated following IL-1β stimulation. A 1.1 kb and 0.6 kb mPGES-1 promoter fragment were generated by PCR and cloned into a human growth hormone (hGH) reporter construct. The human GH reporter gene is a complete genomic locus with introns and exons, producing hnRNA followed by normal splicing events. Moreover, hGH mRNA is known to have a relatively long half-life (12 – 18 h) so assessment of the mRNA by northern blot or real-time RT-PCR is not subject to issues of decay. Another advantage of the system is that it allows for the direct measurement of transcription by evaluating mRNA levels rather than detecting the levels of protein activity.

HFL-1 cells were transiently transfected with each construct and total RNA was analyzed by northern blot for growth hormone expression. The diagram in Figure 3-8(A) depicts the two mPGES-1 promoter/reporter constructs. The results in Figure 3-8(B) indicate that in the absence of stimulus, there is basal growth hormone expression with each promoter construct and upon the addition of IL-1β there is a further increase in growth hormone expression. Densitometric analysis of three experiments revealed that the 1.1 kb promoter construct conferred a 2.5 fold increase in promoter activation following IL-1β stimulation, while the 0.6 kb promoter conferred a 1.5 fold increase (data not shown).

Previous studies by Naraba et al. (206) and Moon et al. (207) highlight the importance of the transcription factor Egr-1 in the stimulus-mediated activation of the mPGES-1 promoter. Therefore the binding site for Egr-1 was located within the mPGES-1 1.1 kb promoter construct and subsequently deleted by site-directed mutagenesis. A binding site for C/EBPβ was also identified in the 1.1 kb promoter by computer analysis as indicated in Figure 3-9(A) and also deleted by site-directed mutagenesis. The mutant constructs were transiently transfected into HFL-1 cells and growth hormone expression was analyzed by northern blot. Figure 3-9(B) illustrates the result of the northern blot analysis and reveals that in the absence of Egr-1, both basal and induced growth hormone expression is significantly reduced, while deletion of the C/EBPβ site did not affect the induction and showed an expression pattern similar to that of the wild type promoter.

Analysis of Internal Cis-Acting Elements That May be Involved in Regulating Microsomal PGES-1 Gene Expression

The published studies on the minimal mPGES-1 promoter and our efforts shown in Figure 3-8 and Figure 3-9 demonstrate that although a ~ 2 fold induction is observed following stimulus treatment, the proximal promoter fragments do not recapitulate the steady state increase of ~ 8 to 9 fold (Figure 3-2 and Figure 3-3). Therefore, in an attempt to identify additional potential regulatory elements within the mPGES-1 locus, a series of overlapping fragments across intron 1 to the beginning of exon 3 were generated as indicated in Figure 3-10(A). This strategy was based more on our laboratory's previous experience in identifying internal cytokine-dependent regulatory elements versus an experimental rationale for mPGES-1. The fragments were cloned into the 1.1 kb promoter fragment driving human growth hormone expression and analyzed by transient transfection and northern blot. All fragments were analyzed; whereas Figure 3-10(B) illustrates a representative blot of three fragments, indicating that none of the fragments

conferred a significant increase in growth hormone expression over that of the wild type promoter construct. As such the brute force approach was clearly not adequate to systematically identify relevant regulatory sequences.

Microsomal PGES-1 Chromatin Structure: DNase I Hypersensitive Site Analysis

As an alternative strategy, DNase I hypersensitive analysis was undertaken as an approach that can: (i) scan larger regions for alterations in chromatin structure; (ii) provide a rationale that open chromatin structure or hypersensitive sites would harbor regulatory factors and their analogous binding sites and; (iii) ultimately rapidly identify, although not based on functional significance, regulatory sequences relevant to IL-1β regulation. Located on the long arm of chromosome 9, the mPGES-1 gene spans 15 kb containing two introns and three exons and thus can be effectively studied by DNase I hypersensitive site analysis due to its small size. First, a restriction fragment of at least 10-13 kb was identified, then a single copy probe specific to one end of the fragment was generated by PCR, which would later be used for indirect-end labeling coupled to Southern analysis. DNase I hypersensitive site analysis was performed as described in the Materials and Methods, with HFL-1 cells incubated in the absence or presence of IL-1β. Cells were permeabilized with lyso-lecithin to allow access of DNase I, individual samples were then treated with increasing concentrations of DNase I and total genomic DNA was purified. DNA was cut by restriction digest using an enzyme to define fragments flanking the mPGES-1 locus, size-fractionated on an agarose gel, transferred to a nylon membrane and subjected to Southern analysis using indirect labeling with a single copy probe. This displays any regions of altered chromatin and allows for the direct mapping of these sites based on the indirect end labeling. The diagram in Figure 3-11(A) schematically depicts the position of a 13.3 kb HindIII fragment which spans from -6.4 to +6.8 kb, mapping a region directly 5' to the transcriptional initiation site. Figure 3-11(B) illustrates the result of a hypersensitive site analysis of the

promoter region which revealed the existence of a constitutive hypersensitive site which maps at ~-0.3 kb. This correlates with the location of the Egr-1 binding site in the proximal promoter.

In a similar analysis, an adjacent HindIII fragment spanning from -19.6 to -6.4 kb depicted in Figure 3-12(A) was evaluated. Similar to the proximal promoter DNase I site designated HS1, a second hypersensitive site was identified, present in both control and IL-1β-treated cells. Figure 3-12(B) shows the results for this second hypersensitive site analysis looking further 5' of the promoter and demonstrating the existence of a constitutive hypersensitive site that maps to ~-8.6 kb.

Discussion

Previous reports indicate that mPGES-1 gene expression is up-regulated in response to cytokine treatment in a number of cells and tissues including the lung (207,237,244-247). Whereas, in the absence of stimuli, there is low level basal mRNA and protein expression of endogenous mPGES-1. Presumably this is a consequence of the cells maintaining a homeostatic balance, due to a lack of substrate produced from the upstream activities of PLA_2 and COX enzymes, which does not require the synthesis of downstream synthases such as mPGES-1. Alternatively, in inflammatory situations where systemic/immune cell-derived pro-inflammatory mediators such as IL-1β are elevated, synthesis of signaling molecules, such as prostanoids, are induced to locally initiate events such as vasoconstriction or airway responsiveness.

The data presented in this dissertation illustrates that, in human lung fibroblast cells, mPGES-1 mRNA and protein expression are both induced at high levels following treatment with the pro-inflammatory cytokine, IL-1β. In addition, treatment with the global transcriptional inhibitor actinomycin D blocked the IL-1β induction of mPGES-1 mRNA and evaluation of mPGES-1 heterogeneous nuclear RNA levels following IL-1β stimulation revealed a significant increase in the level of un-spliced message within 1 h of cytokine treatment. Both studies

demonstrate that *de novo* transcription is at least in part required for the IL-1β induction and as an alternative to *de novo* transcriptional events, the stability of the mPGES-1 message could also have an impact on stimulus-dependent increases. The results shown in Figure 3-5 indicate that the mRNA for mPGES-1 has an induced half-life around 6 h. In 2006 Degousee et al. (233) showed that in cardiomyocytes stimulated with IL-1β or LPS in conjunction with actinomycin D, the mPGES-1 mRNA half-life was about 6 h, an observation consistent with our results.

Analysis of mPGES-1 promoter activity by transient transfection revealed approximately a 2 fold increase in expression following IL-1β treatment. Naraba et al. (206) identified an Egr-1 consensus site, which is highly similar to the Sp1 binding site, in the mPGES-1 promoter and illustrated the importance of this site by promoter deletion analysis. Similar to their study, the Egr-1 binding site was evaluated in the human mPGES-1 1.1 kb promoter construct used in our work. Deletion of the Egr-1 sequence revealed that loss of Egr-1 binding attenuated promoter activity with a loss of both the basal and induced expression. Further, a computer predicted binding site for the transcription factor, C/EBPβ was identified within our 1.1 kb promoter construct and deletion of this site did not appear to have an effect on the basal or induced expression of the promoter. Together these studies revealed the importance of Egr-1 in basal and induced promoter activation but the proximal promoter alone did not recapitulate the level of induction seen by northern analysis of endogenous mPGES-1 gene expression stimulated with IL-1β.

We have shown that endogenous expression of mPGES-1 is induced 8-10 fold by IL-1β but activation of the promoter by Egr-1 only generates a ~2 fold increase in mPGES-1 expression. Therefore, the assumption that potential regulatory elements exist outside of the proximal promoter region could account for the observed increase in endogenous expression by

IL-1β. In our attempt to analyze the mPGES-1 locus, overlapping fragments 3' to the start of transcription were evaluated in context of the human mPGES-1 1.1 kb promoter construct. None of the fragments analyzed were able to elicit an increase in the IL-1β induction similar to that of endogenous mPGES-1 mRNA. Furthermore, each fragment behaved similar to the proximal promoter and only generated a ~1.5 – 2 fold increase in promoter activity.

DNase I hypersensitive analysis can be used to detect subtle changes in chromatin structure and for scanning large regions of DNA. The alterations in chromatin structure are known to be associated with binding of regulatory factors and gene transcription, thus this method was employed in our next study. There are inducible and constitutive DNase I hypersensitive sites, both of which are associated with transcriptional activation. Our results identified two constitutive hypersensitive sites. The first site actually mapped to the proximal promoter region, ~ 0.3 kb and the Egr-1 site which was functionally analyzed in this chapter.

The second site, HS2, is also a constitutive hypersensitive site and maps further 5' of the promoter at ~8.6 kb. Although this site is also constitutive, a finer analysis may illustrate the existence of regulatory elements within this site that could possibly account for the regulation of mPGES-1 expression through inducible binding of transcription factors but which cannot be observed at the level of this chromatin study. In Chapter 4, the HS2 site will be further analyzed in an attempt to identify elements involved in the regulation of mPGES-1 gene expression by IL-1β.

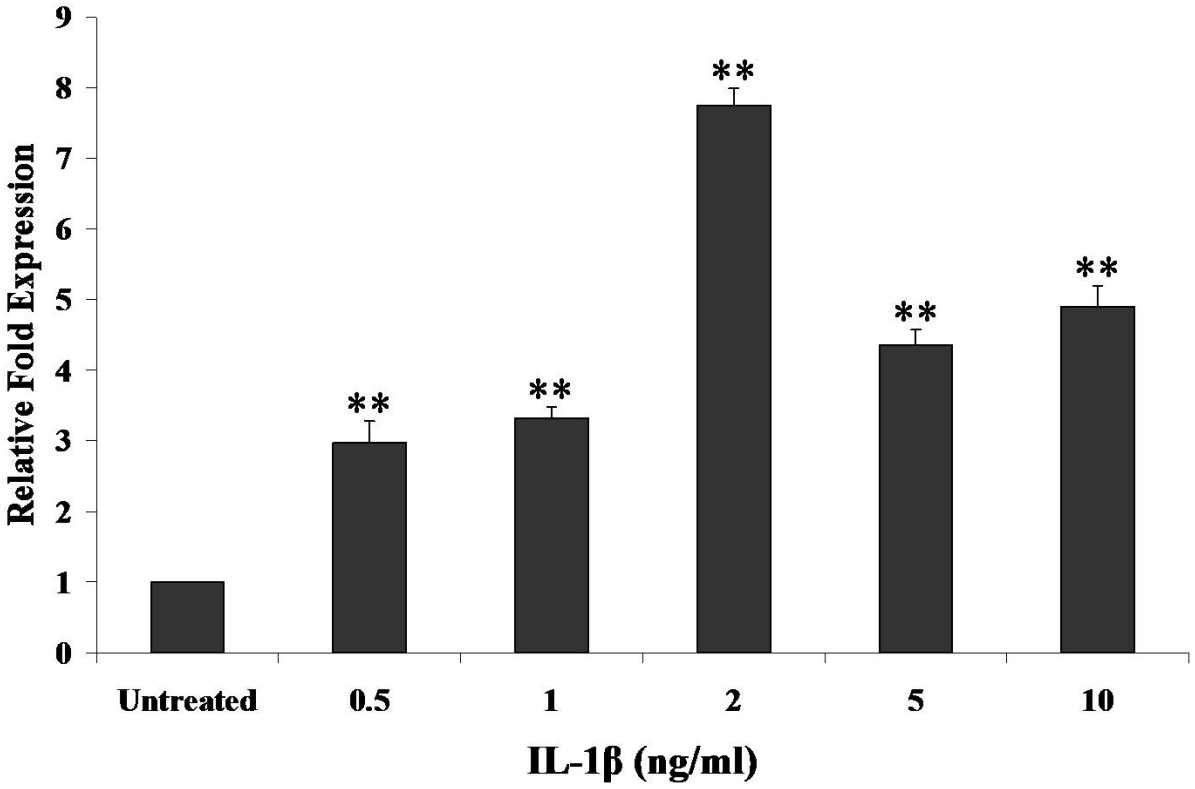

Figure 3-1. Induction of mPGES-1 gene expression by the pro-inflammatory cytokine, IL-1β in human lung fibroblasts. HFL-1 cells were treated with increasing concentrations of IL-1β for 8 h and total RNA was analyzed by real-time PCR for mPGES-1 mRNA expression. The graph depicts three independent experiments and the asterisk (**) indicates statistical significance with p value $\leq$ 0.01 compared to the untreated sample.

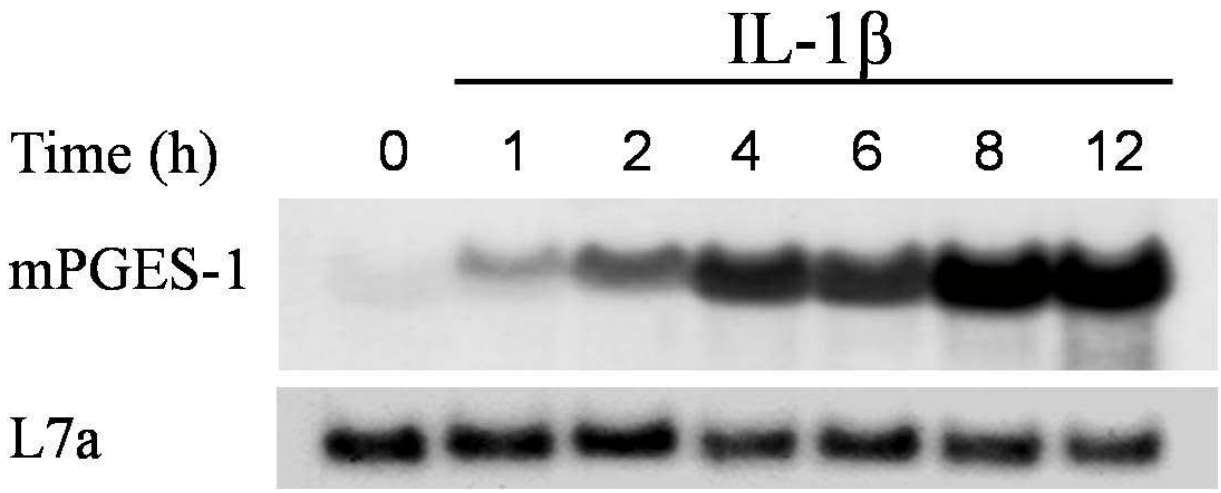

Figure 3-2. Induction of mPGES-1 mRNA expression by IL-1β in human lung fibroblasts. HFL-1 cells were treated with or without IL-1β over the course of 12 h. Total RNA was extracted and analyzed by northern blot. The membrane was hybridized with radiolabeled probes for mPGES-1 and L7a (L7a serves as the loading control).

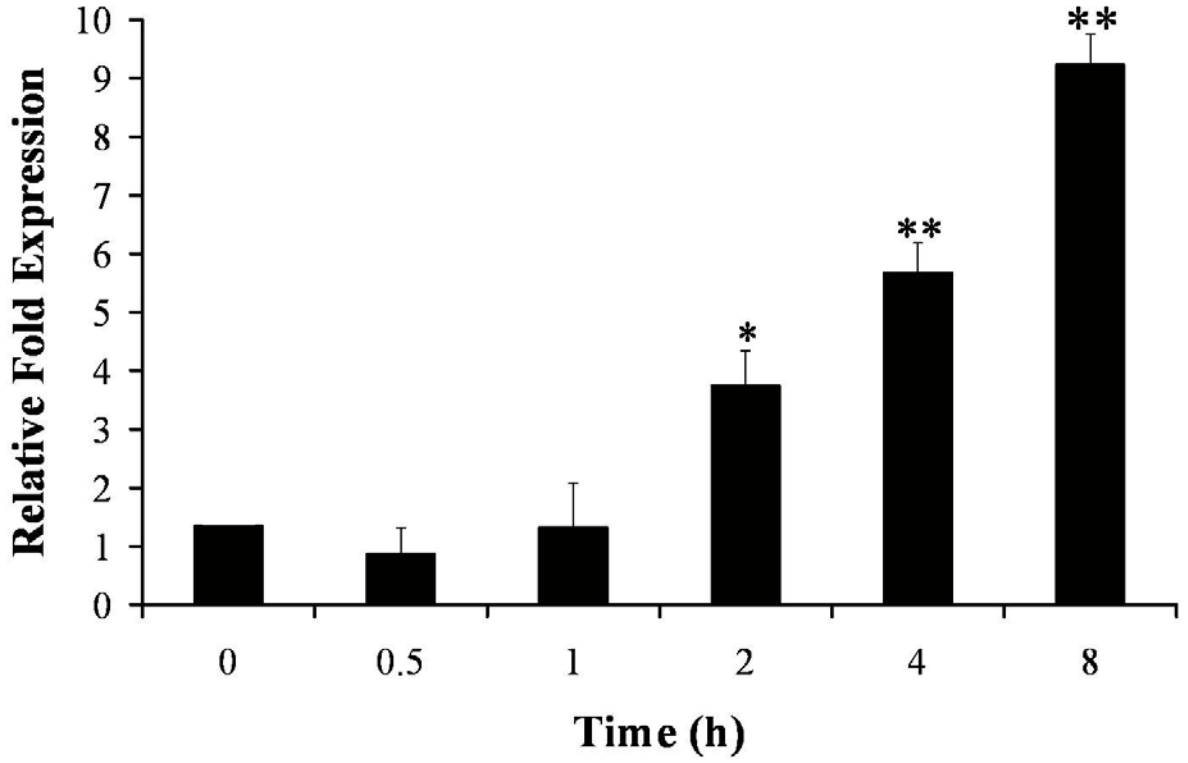

Figure 3-3. Induction of mPGES-1 mRNA expression by IL-1β in human lung fibroblasts: HFL-1 cells analyzed by quantitative real-time RT-PCR analysis. HFL-1 cells were treated with or without IL-1β over the course of 8 h. Total RNA was extracted and subjected to real-time RT-PCR to determine mPGES-1 and cyclophilin A mRNA levels. The mPGES-1/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of three independent experiments, where the data points are represented as mean ± SEM (standard error of the mean). The asterisk (*) indicates statistical significance with p value $\leq$ 0.05 and (**) indicates p value $\leq$ 0.01 as compared with the control sample.

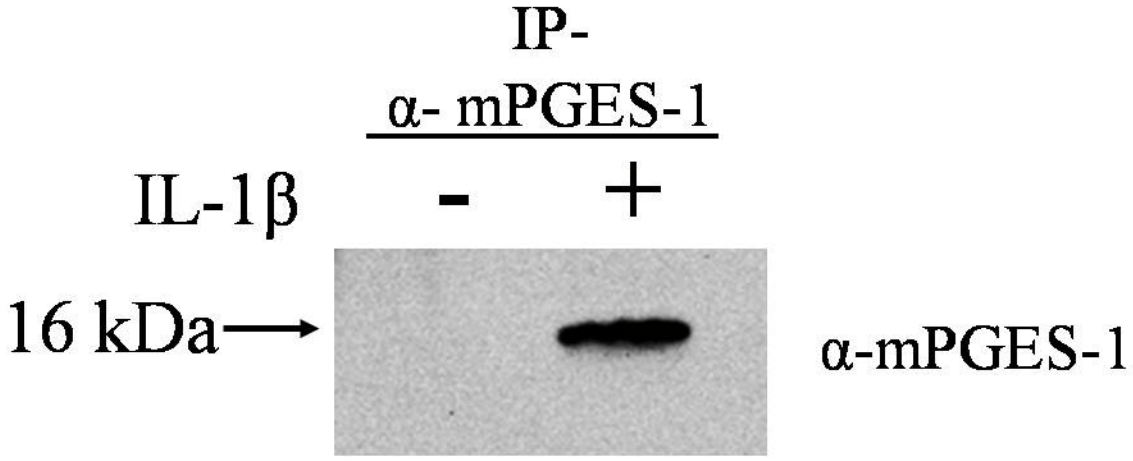

Figure 3-4. Induction of mPGES-1 protein expression by IL-1β in human lung fibroblasts. HFL-1 cells were stimulated with IL-1β for 72 h, total protein was isolated and immunoprecipitated with a monoclonal antibody against mPGES-1. Immunoblot analysis was conducted with a polyclonal antibody against mPGES-1

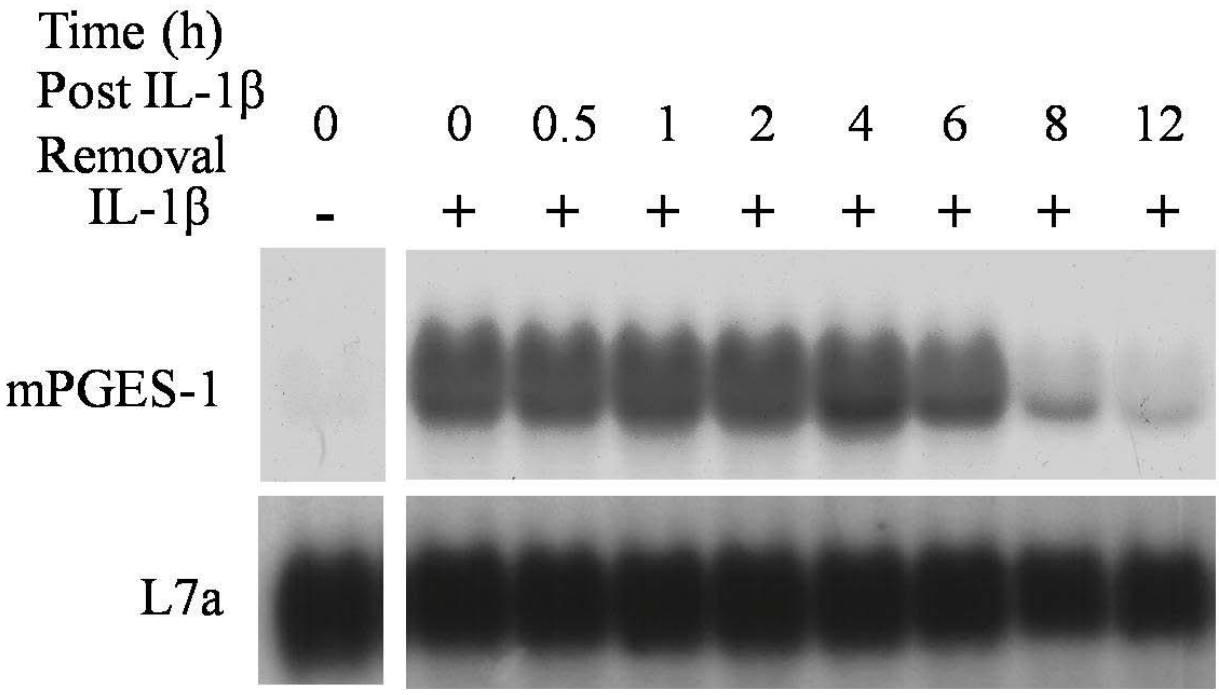

Figure 3-5. Determination of mPGES-1 mRNA decay following stimulus removal. HFL-1 cells were stimulated with IL-1β for 8 h; the stimulus was removed and fresh media was added to each plate. The cells were lysed at the indicated times, total RNA extracted and analyzed by northern blot. The membrane was hybridized with radiolabeled probes for mPGES-1 and L7a.

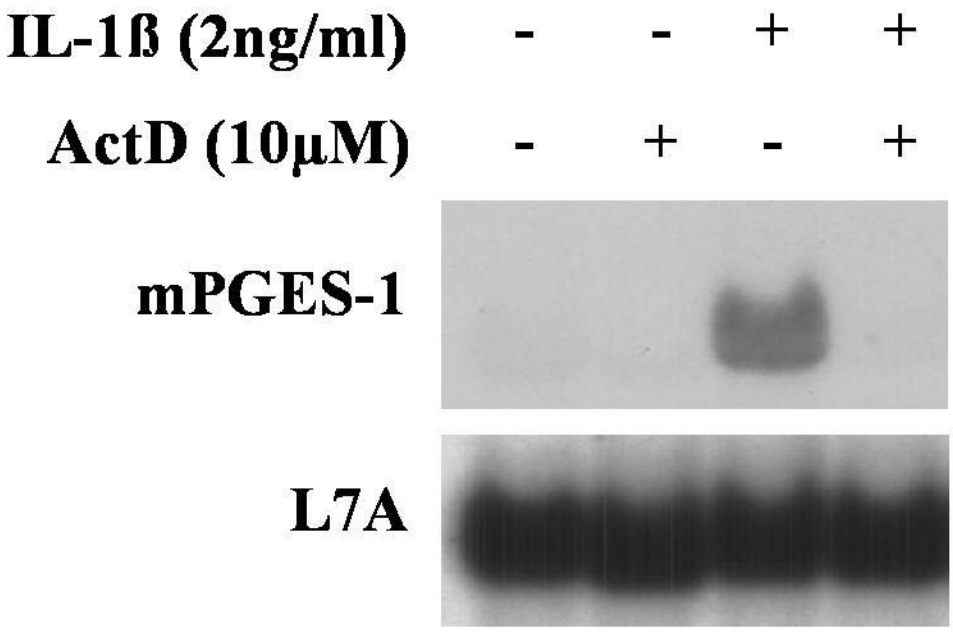

Figure 3-6. The IL-1β induction of mPGES-1 gene expression requires *de novo* transcription. HFL-1 cells were treated with actinomycin D in the absence or presence of IL-1β. At the indicated time points, total RNA was isolated and analyzed by northern blot. The membrane was hybridized with radiolabeled probes for mPGES-1 and L7a (L7a serves as the loading control).

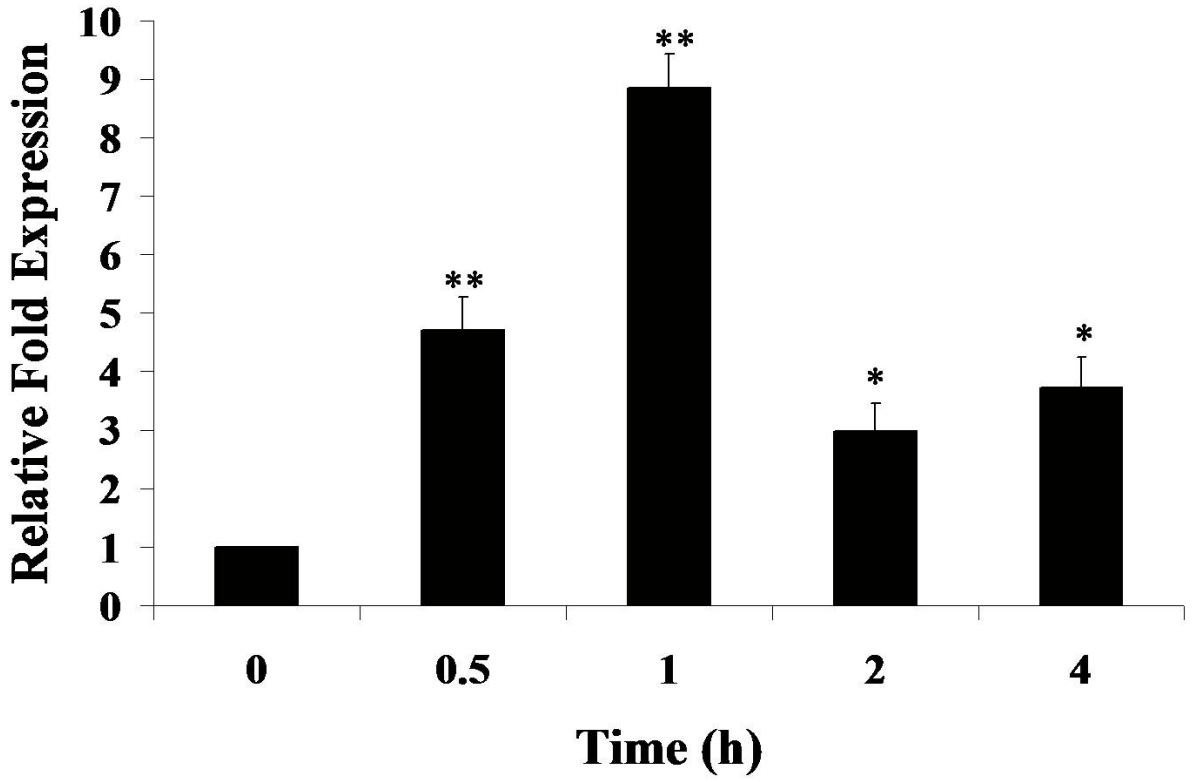

Figure 3-7. The IL-1β induction of mPGES-1 gene expression requires de novo transcription: Analysis of hnRNA levels. HFL-1 cells were stimulated with IL-1β for the indicated times and total RNA isolated then subjected to real-time RT-PCR analysis to detect mPGES-1 hnRNA levels. The mPGES-1 hnRNA/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of three independent experiments, where the data points are represented as mean ± SEM (standard error of the mean). The asterisk (*) indicates statistical significance with p value $\leq$ 0.05 and (**) indicates p value $\leq$ 0.01 as compared with the control sample.

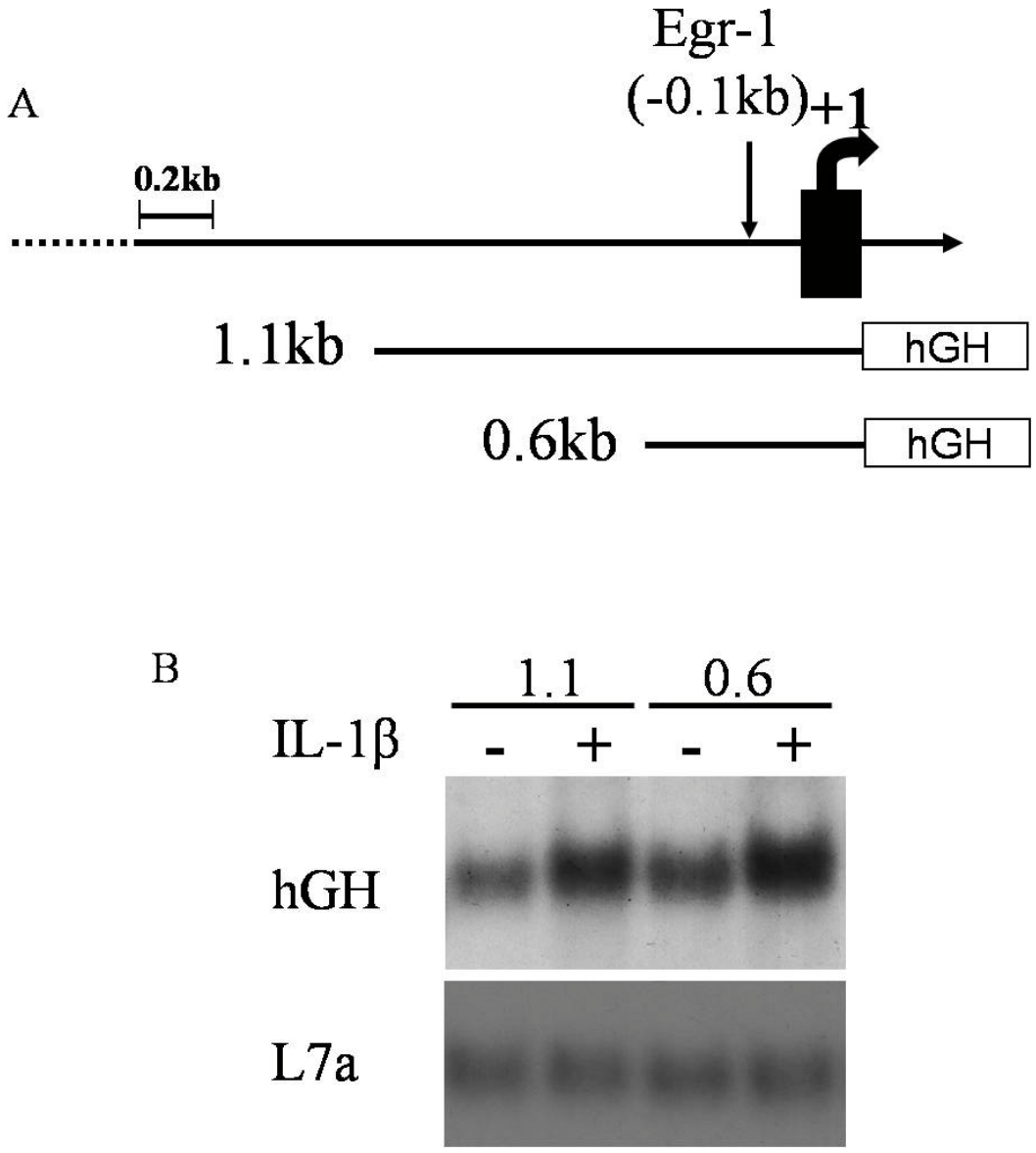

Figure 3-8. Evaluation of the mPGES-1 proximal promoter. A) A depiction of the mPGES-1 promoter fragments driving the expression of human growth hormone (hGH) reporter. B) HFL-1 cells were transiently transfected with either the 1.1 kb or the 0.6 kb promoter construct and 40 h post transfection cells were either untreated or stimulated with IL-1β for 8 h. Total RNA was extracted and analyzed by northern blot. The membrane was hybridized with radiolabeled probes for hGH and L7a.

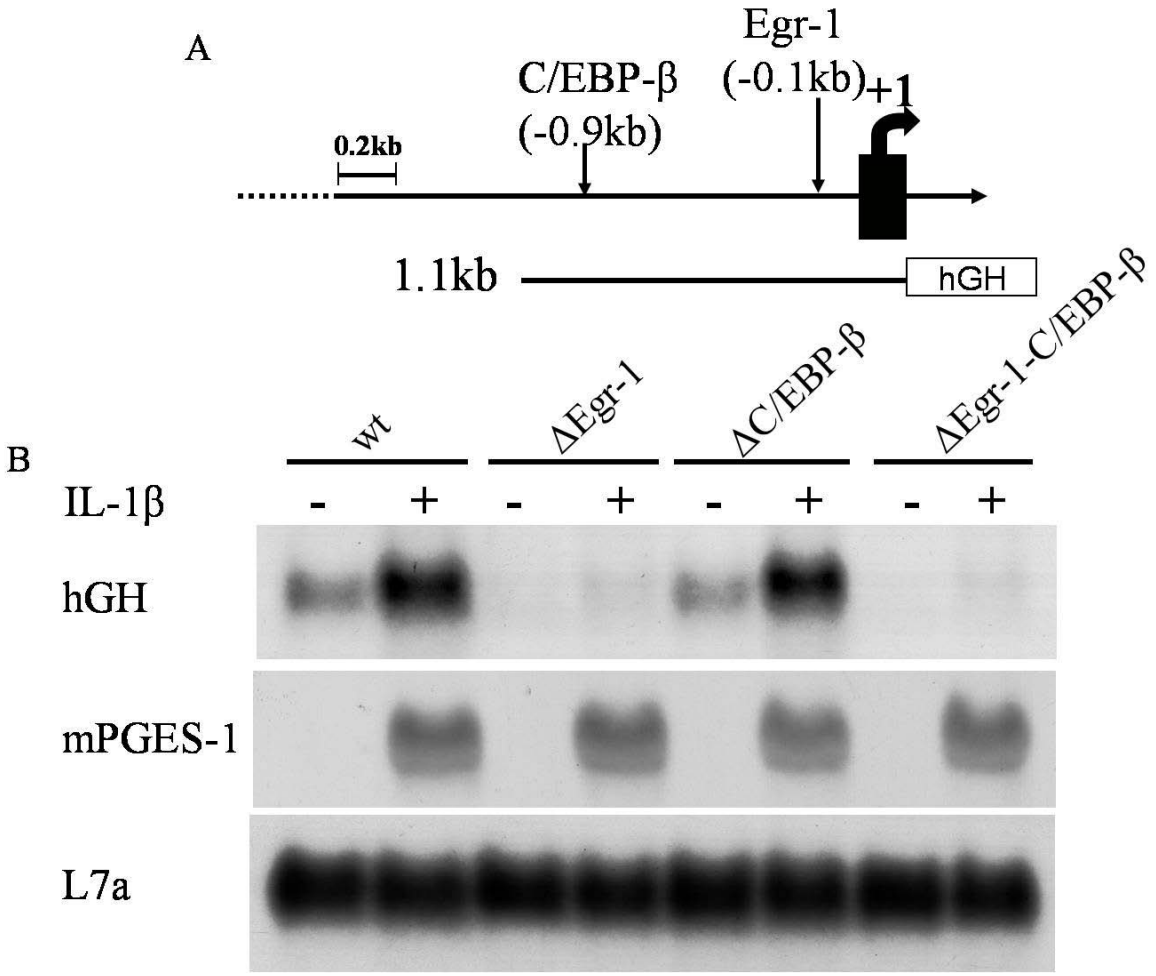

Figure 3-9. Evaluation of the mPGES-1 proximal promoter. A) A schematic of the 1.1 kb promoter fragment indicating the location of transcription factor binding sites for Egr-1 (-0.1 kb) and C/EBPβ (-0.9 kb) relative to the start of transcription (+1). B) The binding sites of Egr-1 and C/EBPβ were deleted (ΔEgr-1 and ΔC/EBPβ) from the 1.1 kb promoter fragment construct by site-directed mutagenesis. Each construct was transiently transfected into HFL-1 cells and 48 h later, total RNA was extracted from control and IL-1β treated cells, then analyzed by northern blot. The membrane was hybridized with radiolabeled probes for hGH, mPGES-1 and L7a.

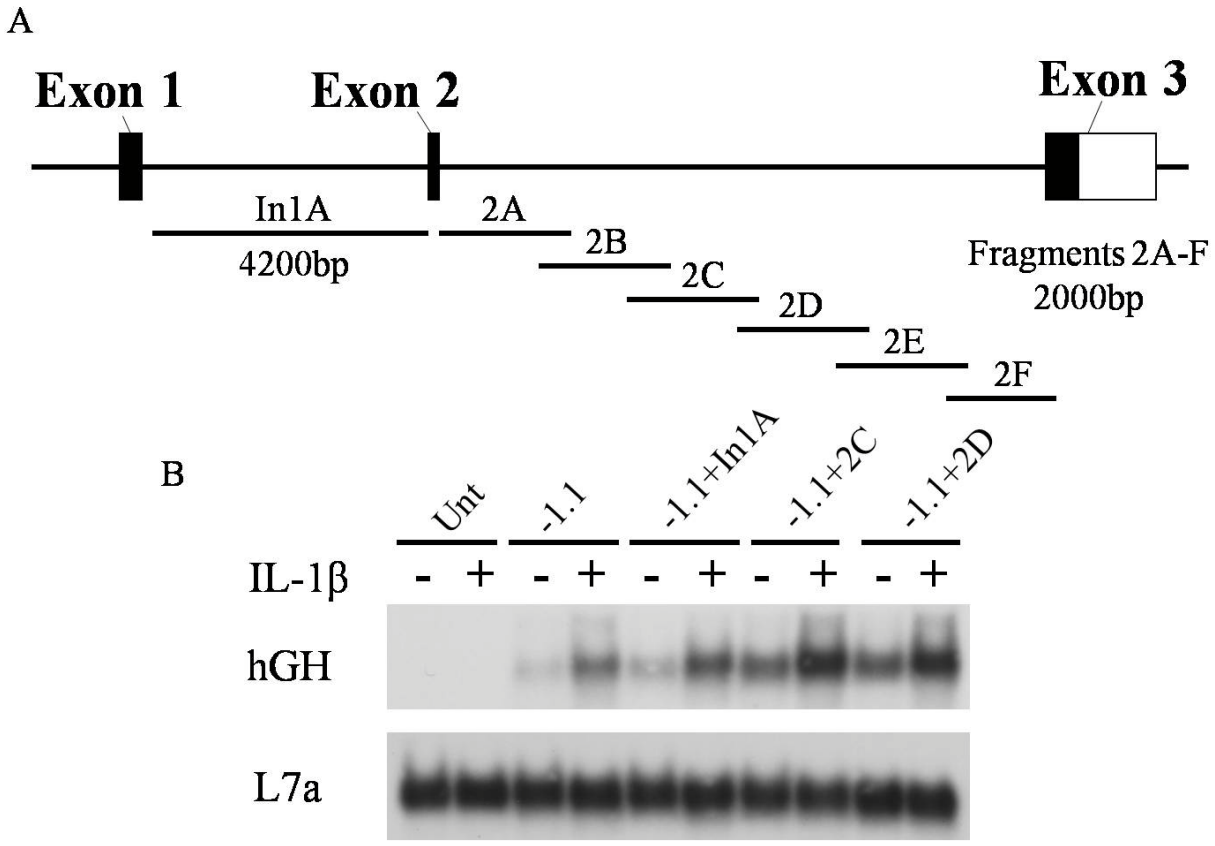

Figure 3-10. Analysis of internal cis-acting elements that may be involved in regulating mPGES-1 gene expression. A) A series of overlapping fragments spanning from the beginning intron 1 to the beginning of exon 3 were generated by PCR and cloned into the 1.1 kb mPGES-1 promoter construct driving hGH expression. B) HFL-1 cells were transiently transfected with the indicated construct and total RNA was isolated from untreated or cytokine treated plates then analyzed by northern blot. The membrane was hybridized with radiolabeled probes for hGH and L7a.

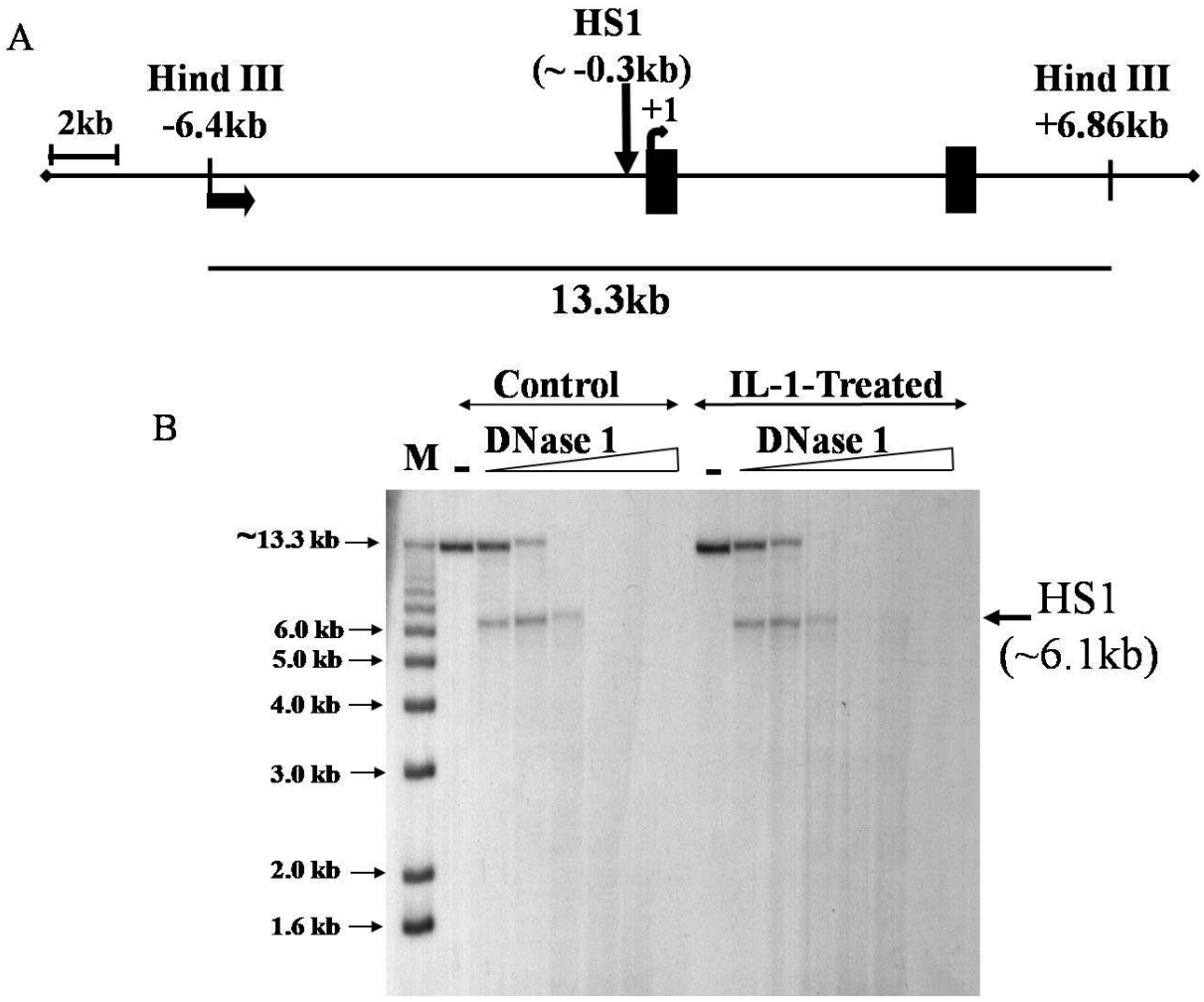

Figure 3-11. mPGES-1 chromatin structure: DNase I hypersensitive site analysis 1. A) A diagram of the mPGES-1 gene, indicating the location of a 13.3 kb HindIII fragment covering -6.4 to +6.8 kb. The dark arrow (➡) at the front of the 13.3 kb line indicates the location of a single copy probe abutting the 5'end of the HindIII restriction site which was used for indirect end-labeling of the genomic restriction fragment as well as hybridizing to HS1 located at ~-0.3 kb. B) Southern blot analysis of a 13.3 kb fragment to detect HS sites within the mPGES-1 promoter region. M denotes the molecular weight markers and the (-) lane indicates the genomic HindIII fragment with no DNase I treatment. The triangle denotes increasing concentrations of DNase I in control and cytokine treated cells. The arrow on the right side indicates the approximate size of the HS1 site (~6.1 kb) which maps to the proximal promoter region of the mPGES-1 gene.

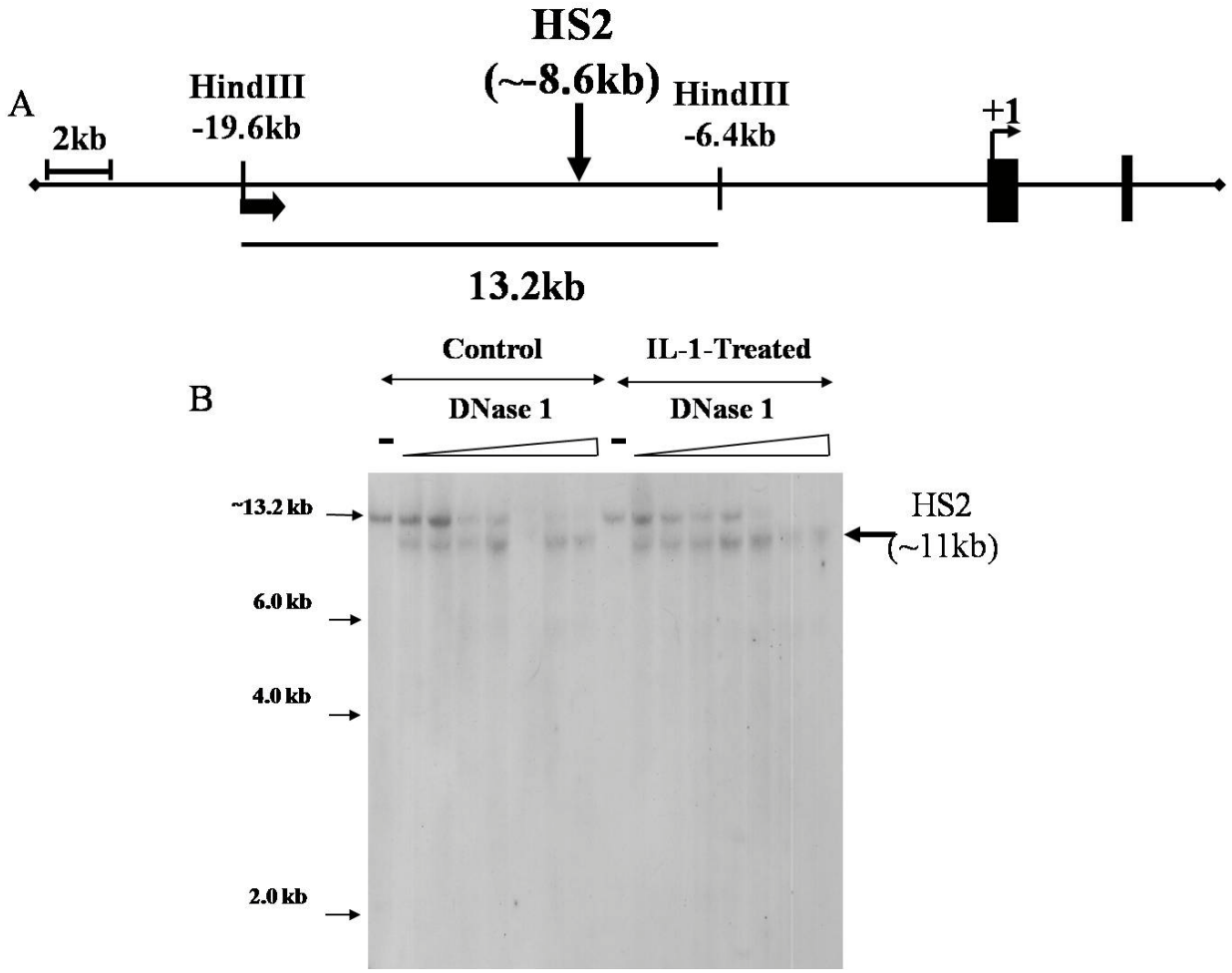

Figure 3-12. mPGES-1 chromatin structure: DNase I hypersensitive site analysis 2. A) A depiction of the mPGES-1 gene, indicating the location of a 13.2 kb HindIII fragment covering -19.6 to -6.4 kb. The dark arrow (➡) at the front of the 13.2 kb line indicates the location of a single copy probe abutting the 5'end of the HindIII restriction site which was used for indirect end-labeling of the genomic restriction fragment as well as hybridizing to HS1 located at ~-8.6 kb. B) Southern blot analysis of a 13.2 kb fragment to detect HS sites within the mPGES-1 genome. The (-) lane indicates the genomic HindIII fragment with no DNase I treatment. The triangle denotes increasing concentrations of DNase I in control and cytokine treated cells. The arrow on the right side indicates the approximate size of the HS1 site (~11 kb) which maps to the distal promoter region of the mPGES-1 gene.

CHAPTER 4
FUNCTIONAL ANALYSIS OF PROMOTER AND DISTAL REGULATORY ELEMENTS CONTROLLING THE IL-1β INDUCTION OF MICROSOMAL PROTAGLANDIN E SYNTHASE-1 (MPGES-1) GENE EXPRESSION

Introduction

Gene transcription is commonly associated with remodeling of chromatin structure. In the inactive state, DNA is tightly associated with nucleosomes and maintained as heterochromatin; virtually prohibiting the binding of transcription factors to the DNA. When gene transcription is activated, the nucleosomes are modified allowing access to DNA and subsequent binding of transcription factors and the general transcription machinery. The balance between gene silencing and activation is known to be mediated by the action of histone deacetylases and acetyltransferases, respectively (248-252).

During gene transcription, DNA is also more susceptible to DNase I digestion leading to the detection of "hypersensitive" sites. There are two types of hypersensitive sites, constitutive and inducible. In constitutive sites, DNA is held open and free of nucleosomes, independent of a stimulus. These sites are normally associated with the promoter region of genes poised for transcriptional activation (253-255). On the other hand, for inducible sites, DNA and nucleosomes are tightly associated and upon addition of stimulus the chromatin structure is modified, the nucleosomes are removed allowing access to the DNA, which is now sensitive to DNase I cleavage (254,255). These hypersensitive sites are thought to be devoid of nucleosomes and usually found within the 5' region of genes, close to or within regions that are involved in regulating gene expression. Numerous studies of the human β-globin locus control region revealed the presence of enhancer elements within hypersensitive sites that are known to control gene expression (256-258). Enhancers elements are known to act in both a position and orientation independent manner and in the context of a heterologous promoter (259).

As shown in Chapter 3, published data and the results presented thus far, implicate the need for additional elements to explain the level of cytokine-mediated induction. The data presented in Figure 3-12 indicated the existence of a DNase I hypersensitive site mapping at ~8.6 kb, 5' to the transcription initiation site. Although it is a constitutive site, analysis of this region for functional activity seemed to be the next logical approach.

Results

Functional Analysis of the Distal Hypersensitive Site, HS2 Relative to the Microsomal PGES-1 Promoter

In the previous chapter two DNase I hypersensitive sites were identified within the mPGES-1 genome, one mapping to the proximal promoter region at the Egr-1 binding site and the other in the distal 5' region of the promoter (~8.6 kb). Since the first hypersensitive site mapping to the proximal promoter region was previously evaluated in the proximal promoter constructs, the distal hypersensitive site, HS2, will now be evaluated in context with the mPGES-1 promoter. A fragment spanning HS2 region, -10.7 to -6.4 kb, was amplified by PCR and cloned in front of the 1.1 kb promoter construct driving human growth hormone expression. Activity of the construct was then evaluated by transient transfection and northern blot analysis in HFL-1 cells. Figure 4.1 (A and B) illustrates the location of the -10.7 to -6.4 kb fragment around the HS2 site and the results of the transfection. In the presence of the -10.7 to -6.4 kb fragment, there was a significant increase in basal growth hormone expression compared to the wild type construct. Treatment with IL-1β caused a further increase in growth hormone expression with the HS2 containing construct. The membrane was also reprobed for mPGES-1 expression demonstrating the normal level of IL-1β induction. Also, comparing the un-induced lane for the promoter alone (1.1 kb) with the induced lane in the HS2 construct (1.1 + (-10.7 to -6.4)), clearly illustrates a comparable level of induction to the endogenous gene. A graph

depicting densitometry of three independent experiments is shown in Figure 4-2 and illustrates that the IL-1β treated cells display an overall induction of approximately 9 fold if compared back to the untreated promoter alone samples normalized to 1.

HS2 Exhibits Characteristics of an Enhancer: Evaluation of HS2 Using a Minimal Thymidine Kinase Heterologous Promoter

The fragment was then analyzed for enhancer activity. This was accomplished by subcloning the -10.7 to -6.4 kb fragment into a human growth hormone reporter construct containing a 0.2 kb minimal viral thymidine kinase promoter fragment. This experiment would demonstrate the ability of this region to work with a heterologous promoter in a stimulus dependent manner. Figure 4-3 (A and B) illustrates the thymidine kinase promoter construct and the results of the northern blot analysis. The TK promoter construct alone exhibited no significant increase in promoter activity following IL-1β stimulation. The presence of the -10.7 to -6.4 kb fragment slightly increased basal growth hormone expression with a further increase in growth hormone expression following IL-1β treatment. A graph depicting densitometry of three independent experiments is shown on the left of Figure 4-4.

To further address that HS2 functions as an enhancer, the thymidine kinase promoter construct containing the -10.7 to -6.4 kb fragment in the reverse orientation was evaluated by real-time PCR following transient transfection in HFL-1 cells. A true enhancer can be defined as an element or DNA sequence that functions independent of position or orientation. Briefly HFL-1 cells were transiently transfected with the each construct, total RNA was isolated according to the Qiagen™ RNeasy Mini Kit, DNase treated, then subjected to real-time RT-PCR analysis with growth hormone specific primers. The results on the right of Figure 4-4 show that in the reverse orientation, the HS2 fragment still elicits a strong growth hormone expression following IL-1β treatment, with an induction in either orientation of ~4 – 5 fold. Furthermore, these results

demonstrate that this region of the mPGES-1 locus can function with a heterologous promoter in an orientation independent manner.

Identification of a Basal Element Within HS2

The present results imply that the HS2 fragment can exhibit both basal and inducible activity. Therefore in an attempt to delineate the location of two potential elements, one involved in basal expression and the other, inducible activity, a series of 5' overlapping fragments were generated then cloned into the 1.1 kb promoter construct shown in Figure 4-5(A). At this point it should be noted that the function of transiently transfected plasmid constructs is not controlled by events which mediate responses at the level of chromatin since these plasmid molecules lacked endogenous chromatin structure.

Each construct was evaluated by transient transfection and real-time RT-PCR for growth hormone expression. The diagram in Figure 4-5 depicts the location of the 5' overlapping fragments generated from the HS2 containing fragment, -10.7 to -6.4 kb. The results illustrate that compared to the wild type promoter; the -10.7 to -9.6 kb and -10.1 to -9.0 kb fragments both exhibited an increase in basal growth hormone expression, with a further increase upon IL-1β treatment that was comparable to the induction at the promoter. The last fragment, -9.5 to -8.4 kb, displayed expression levels similar to the 1.1 kb promoter alone. The interpretation of these results is that the large increase in basal expression (~4 -5 fold) observed most prominently with the -10.1 to 9.0 kb fragment indicates the presence of a DNA sequence which may aid in basal expression. However, the level of induction seen in these constructs does not appear to be greater than the promoter alone. Therefore, our hypothesis is that an additional stimulus-dependent element must reside elsewhere in the -10.7 to -6.4 kb enhancer fragment.

Mapping of an Inducible Element Contained Within HS2

An element controlling basal activity has now been mapped to the 5' portion of the HS2 fragment; the next step was to evaluate the remaining DNA sequence for an inducible element. A series of 3' deletion fragments were amplified by PCR and cloned into the 1.1 kb promoter construct. Each construct was evaluated for growth hormone expression following transient transfection and real-time RT-PCR as described in the Materials and Methods. The graph in Figure 4-6 illustrates the results of the real-time RT-PCR analysis and indicates that compared to the wild type promoter, both the -8.6 to -6.4 kb and the -8.6 to -8.1 kb fragment exhibit a significant increase in growth hormone expression following IL-1β treatment. On the other hand, the -8.1 to -7.6 kb and the -7.6 to -6.4 kb fragments displayed a behavior that was similar to that of the wild-type promoter construct.

Overall, this data demonstrates the existence of an element (-8.6 to -8.1 kb) in conjunction with the endogenous promoter, that confers an IL-1β-dependent induction of ~6 - 7 fold, which is comparable to the level of induction seen with the endogenous gene (Figure 3-2). In addition, taking into account the effects of the basal element (Figure 4-5) and the inducible element (Figure 4-6), the combined level of expression recapitulates the natural induction. This further accentuates the importance of multiple regulatory elements, which, when combined with the impact of the endogenous chromatin structure may appropriately recreate the events in the cell.

Identification of Three C/EBPβ Binding Sites in the Distal Regulatory Enhancer Element: Evaluation of Single C/EBPβ Mutants in the IL-1β Induction of Microsomal PGES-1

Having identified an enhancer element covering -8.6 to -8.1 kb which is involved in the IL-1β induction, computer analysis of this 500 bp region was conducted to predict the location of potential transcription factor binding sites using TESS - transcription element search software

(227). Figure 4-7 illustrates the nucleotide sequence of the 500 bp fragment, showing the location of three putative C/EBPβ sites that were identified by TESS analysis.

To verify the relevance of these three C/EBPβ sites to the IL-1β-dependent induction of mPGES-1 expression, each site was deleted by site-directed mutagenesis and evaluated for functional activity by transient transfection and real-time RT-PCR. The results in Figure 4-8 illustrate that deletion of the C/EBPβ Site 1 only, had no effect on the overall induction by IL-1β, while deletion of C/EBPβ Site 3 only, showed a slight increase in the IL-1β induction, over that of both the wild type promoter construct and the non-mutated -8.6 to -8.1 kb construct. The observed increase in the IL-1β induction following deletion of Site 3 is a reproducible trend but was not statistically significant. On the other hand deletion of C/EBPβ Site 2 alone, decreased the IL-1β induction versus the non-mutated -8.6 to -8.1 kb construct yielding an overall induction that was now similar to that of the wild type promoter construct alone.

Analysis of Double C/EBPβ Mutants in the IL-1β Induction of Microsomal PGES-1

To further verify the contribution made by each C/EBPβ site to the overall induction by IL-1β, a series of double mutants were also generated to analyze each individual C/EBPβ site. Figure 4-9 illustrates the following deletions: C/EBPβ Sites 2/3, leaving only Site 1 present, C/EBPβ Sites 1/2, leaving only Site 3 present and C/EBPβ Sites 1/3, leaving only Site 2 present. The deletion of Sites 2/3 and Sites1/2 led to a decrease in the IL-1β induction, similar to that of the promoter alone construct. However, deletion of C/EBPβ Sites 1/3, leaving only Site 2 present led to an increase in overall IL-1β induction over that of the wild type promoter construct coupled to the non-mutated -8.6 to -8.1 kb construct. This a further example that, at least in the context of a plasmid, Site 3 may serve as an additional/competitive binding site since as with the single site mutants the loss of Site 3 and Site 1 showed the similar increase over the unmutated fragment.

Evaluation of the C/EBPβ Sites in Constructs Lacking the Egr-1 Binding Site.

To determine the role Egr-1 binding in the promoter has on the C/EBPβ mediated IL-1β induction seen with the distal enhancer element, another set of C/EBPβ mutant constructs were generated which included deletion of the Egr-1 binding site from the promoter region. Each of these constructs was analyzed by transient transfection in HFL-1 cells and real-time RT-PCR for growth hormone expression. As a comparison, the single site mutant C/EBPβ Δ3 and the double mutant C/EBPβ Δ1/Δ3 constructs were also analyzed. As illustrated in Figure 4-10, absence of Egr-1 binding in either the single site mutant or the double site mutant had virtually no effect on the overall IL-1β induction. This result illustrates that at least in the context of a plasmid molecule, lacking appropriate chromatin structure, the distal C/EBPβ site within the enhancer can strongly and independently drive the IL-1β induction. However, this does not diminish the importance of the Egr-1 site as a relevant activator in the proximal promoter and its role in the endogenous chromatin structure.

Targeted Deletion of C/EBPβ by Short Interfering RNA in Human and Rat Lung Cells

To establish the significance of C/EBPβ with regard to the IL-1β induction of mPGES-1 gene expression, siRNA studies to knockdown C/EBPβ expression were conducted. Both HFL-1 cells and a rat pulmonary epithelial-like cell line, L2, were transfected with siRNAs specifically targeting both human and rat C/EBPβ expression, respectively, and mPGES-1 expression was analyzed by real-time RT-PCR following stimulation with the pro-inflammatory cytokine, IL-1β.

As illustrated in Figure 4-11(A), knockdown of C/EBPβ expression in HFL-1 cells treated with a human C/EBPβ siRNA led to an approximately 60% decrease in the IL-1β-induced expression of endogenous mPGES-1. Knockdown of C/EBPβ expression in HFL-1 cells was verified by immunoblot analysis illustrated in Figure 4-11(B), which shows a decrease in C/EBPβ protein expression. In L2 cells, the results in Figure 4-12 similarly indicate that

knockdown of C/EBPβ expression with a rat specific siRNA led to an approximately 50% decrease in the IL-1β induction following treatment with a rat specific C/EBPβ siRNA.

Evaluation of Microsomal PGES-1 Expression in C/EBPβ Null Mouse Embryonic Fibroblast (MEF) Cells

As further verification of the functional relevance of C/EBPβ, mouse embryonic fibroblasts from C/EBPβ knockout mice were also evaluated to address the role in mPGES-1 gene expression. Wild type or C/EBPβ deficient (C/EBPβ -/-) MEF cells in the absence or presence of IL-1β were evaluated for mPGES-1 mRNA expression by real-time RT-PCR. The results in Figure 4-13 illustrate that while IL-1β induced mPGES-1 mRNA expression in the wild type MEFs, there was no induction of mPGES-1 expression in the C/EBPβ -/- MEFs.

Chromatin Immunoprecipitation (ChIP) Analysis of Egr-1, RNA Polymerase II and C/EBPβ Binding

Binding of Egr-1 to the proximal promoter and C/EBPβ binding to the distal enhancer element following IL-1β treatment were analyzed by ChIP. The results in Figure 4-14 illustrate that Egr-1 is constitutively bound to the promoter with no increased binding following exposure to IL-1β. Binding of RNA Polymerase II to the promoter was also analyzed by ChIP and the data revealed that in the absence of stimulus, RNA Polymerase II is bound at a low level to the promoter and following treatment with IL-1β, there is a significant increase in RNA Polymerase II binding also shown in Figure 4-14. Figure 4-15 illustrates ChIP analysis of the distal enhancer element and the data revealed that in the absence of stimulus, C/EBPβ is a bound to this region while treatment with IL-1β caused a further time-dependent increase in C/EBPβ binding, about ~4 fold higher by 8 h.

Co-Immunoprecipitation Analysis of Egr-1 and C/EBPβ Binding

As previously indicated, the results demonstrate the importance of Egr-1 alone to basal and induced expression in the proximal promoter (Figure 3-9). Similarly, C/EBPβ also plays a

central role as an enhancer specific regulatory factor. Although the mutagenesis studies in Figure 4-10 seem to diminish the overall role of Egr-1 in the context of the enhancer, it was still strongly felt that Egr-1 does have an important role *in vivo*. Therefore, to test this notion, studies were performed to determine whether Egr-1 and C/EBPβ are capable of interacting with each other, as evaluated by co-immunoprecipitation (IP) analysis. Figure 4-16 (A) illustrates that Egr-1 is detectable following immunoprecipitation with an antibody to C/EBPβ. Conversely, C/EBPβ is detectable in a complex when Egr-1 is immunoprecipitated as illustrated in Figure 4-16 (B). An antibody to a histidine tag was employed as a negative control in the IP experiments.

Discussion

In these studies, the presence of a constitutive hypersensitive site, HS2, in the distal promoter region of the mPGES-1 gene was identified. Analysis of a fragment spanning from -10.7 to -6.4 kb encompassing this hypersensitive site, revealed the existence of both a basal and inducible element which contribute to the overall induction by IL-1β and recapitulates the ~8-10 fold expression seen by real-time analysis of endogenous mPGES-1 expression following IL-1β treatment.

The -10.7 to -6.4 kb fragment was evaluated for enhancer-like characteristics and it was found that the fragment is capable of activating gene transcription in an orientation independent manner and with a heterologous promoter. Based on deletion analysis of the 5' end of the HS2 containing fragment, the location of a region conferring basal activity was also delineated. Further analysis of the 3' end of the HS2 containing fragment led to the identification of a 500bp fragment associated with the inducible activity. Computer analysis and subsequent site directed mutagenesis of this IL-1β responsive fragment led to the identification of a C/EBPβ site that is involved in the IL-1β induction.

Moon et al. (207) previously reported that the transcription factor, Egr-1, is required for the IL-1β-dependent induction of mPGES-1 gene expression. As a consequence of this, the contribution of Egr-1 binding at the promoter and C/EBPβ at the enhancer following IL-1β treatment was evaluated for activation of the inducible fragment. Consequently, in the absence of the Egr-1 site, no significant change in the overall induction by IL-1β was observed.

The use of siRNAs to knockdown C/EBPβ illustrated the importance of C/EBPβ expression in regulating mPGES-1 gene expression independent of cell type. As further verification of the involvement of C/EBPβ in regulating the IL-1β induction of mPGES-1, MEF cells deficient for C/EBPβ were utilized. It was found that while IL-1β increased mPGES-1 mRNA levels approximately 2 fold in wild type MEFs, in C/EBPβ -/- MEFs IL-1β treatment attenuated mPGES-1 gene expression, further validating the importance of C/EBPβ in the IL-1β induction.

Previous deletion analysis of the proximal promoter region confirmed that Egr-1 binding is involved in the inducible expression of mPGES-1 (206,234). Chromatin immunoprecipitation analysis showed that under basal conditions Egr-1 was already bound to the promoter and further addition of IL-1β did not increase Egr-1 binding. The ChIP data also represents the first study of Egr-1 binding by ChIP analysis. The data also revealed RNA Polymerase II was bound to the promoter under basal conditions and treatment with IL-1β caused a significant increase in RNA Polymerase II binding.

Analysis of Egr-1 binding and RNA Polymerase II binding to the enhancer element revealed no significant binding prior to and following IL-1β treatment (data not shown). Evaluation of C/EBPβ binding to IL-1β responsive element by chromatin immunoprecipitation revealed that C/EBPβ was initially bound and significantly increased following IL-1β treatment.

A search of the current literature yielded no studies evaluating whether Egr-1 and C/EBPβ are capable of interacting. Co-immunoprecipitation of Egr-1 and C/EBPβ followed by immunoblot analysis revealed that Egr-1 and C/EBPβ do interact but in an IL-1β independent manner. The model in Figure 4-17 illustrates that low levels of C/EBPβ and RNA Polymerase II are bound to the mPGES-1 locus in the absence of stimulus. Following IL-1β treatment, there is increased RNA Polymerase II and C/EBPβ binding at the promoter and enhancer, respectively. Egr-1 binding was constitutively observed at the promoter potentially leading to cross-talk between Egr-1 and C/EBPβ and subsequent activation of mPGES-1 gene expression.

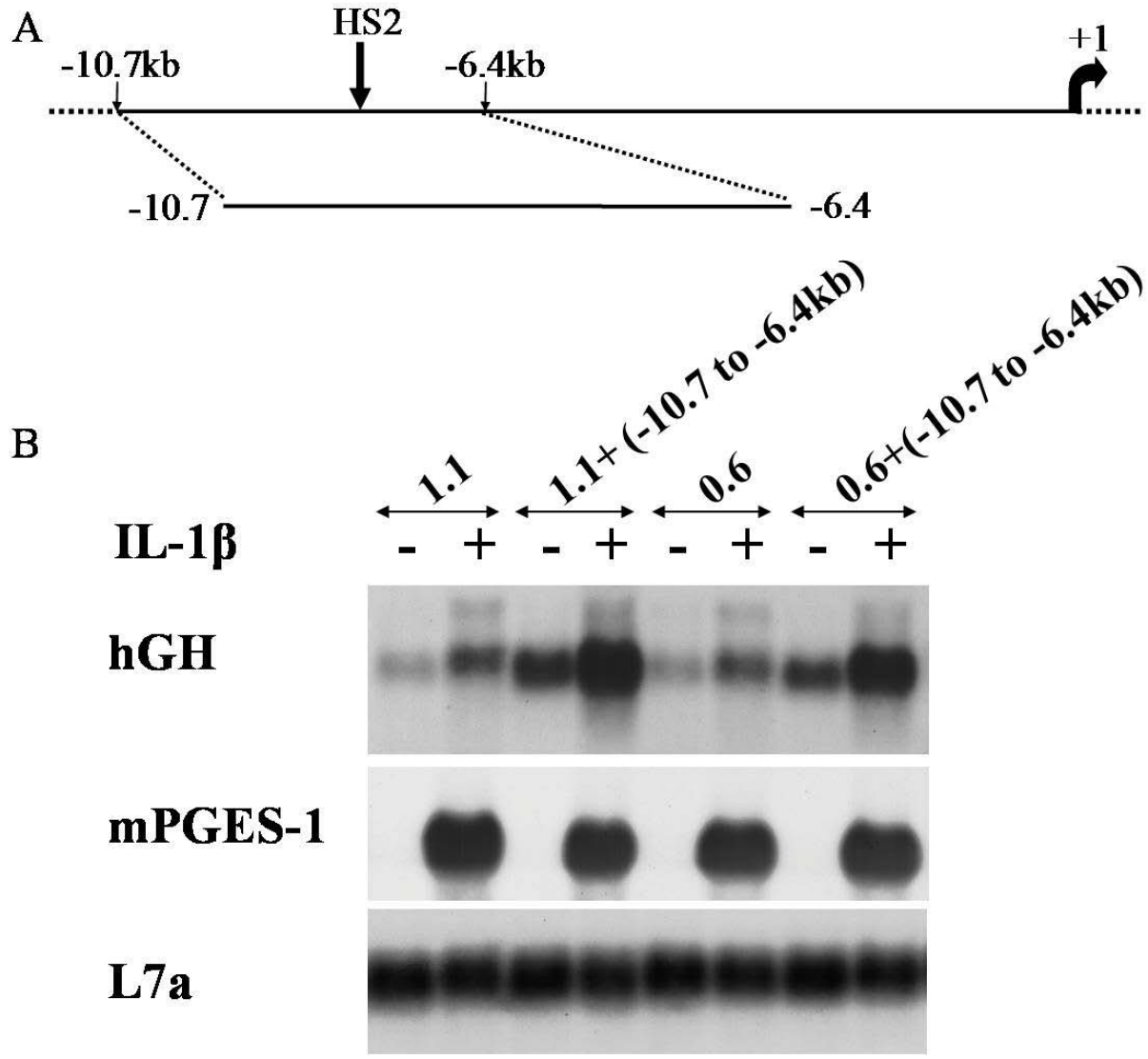

Figure 4-1. Functional analysis of the distal hypersensitive site, HS2 relative to the mPGES-1 promoter. A) A schematic of the HS2 containing fragment -10.7 to -6.4 kb. B) The -10.7 to -6.4 kb fragment was cloned into the 1.1 kb or 0.6 kb promoter construct driving hGH expression. HFL-1 cells were transiently transfected with each construct, total RNA was extracted and analyzed by northern blot. The membrane was hybridized with radiolabeled probes for hGH, mPGES-1 and L7a.

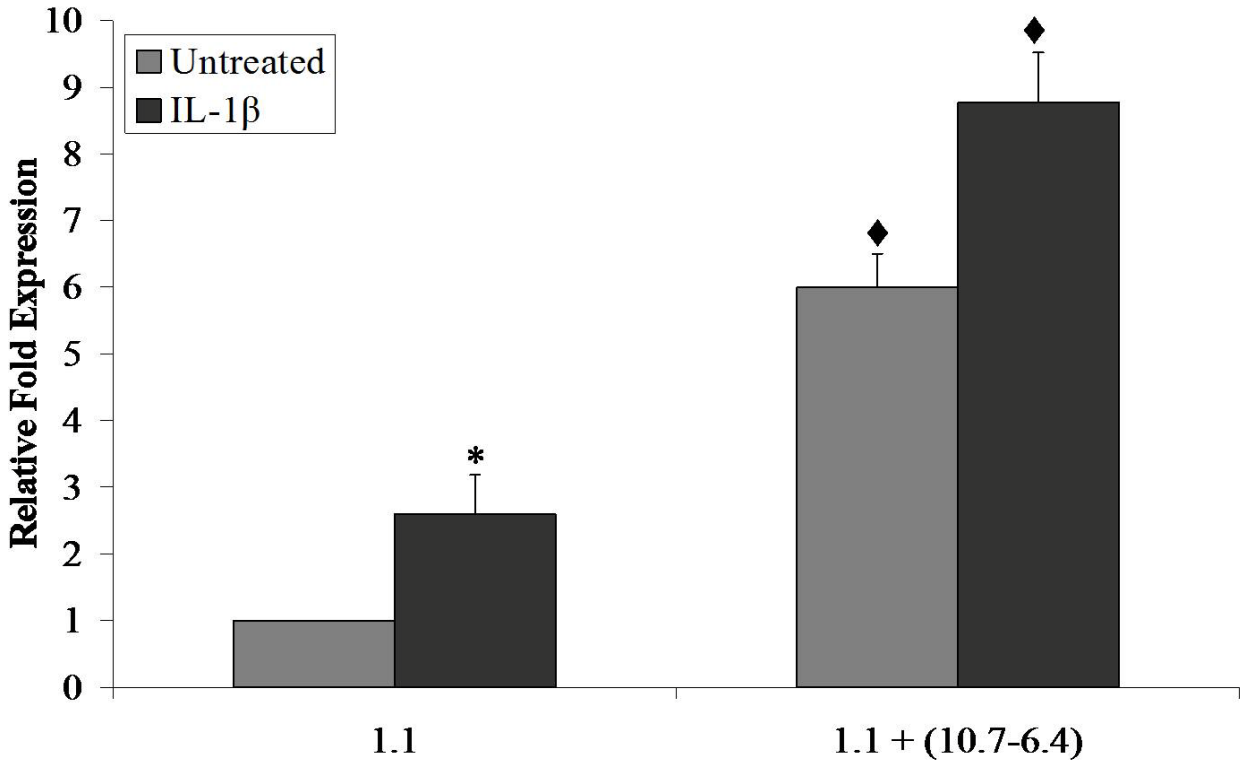

Figure 4-2. Functional analysis of the distal hypersensitive site, HS2 relative to the mPGES-1 promoter. The graph illustrates densitometry of three independent northern blot analyses where data points are represented as mean ± SEM. The asterisk (*) denotes statistical significance with p value ≤ 0.05 compared with the untreated wild type promoter. The diamond (♦) denotes statistical significance with p value ≤ 0.05 as compared with the untreated wild type promoter.

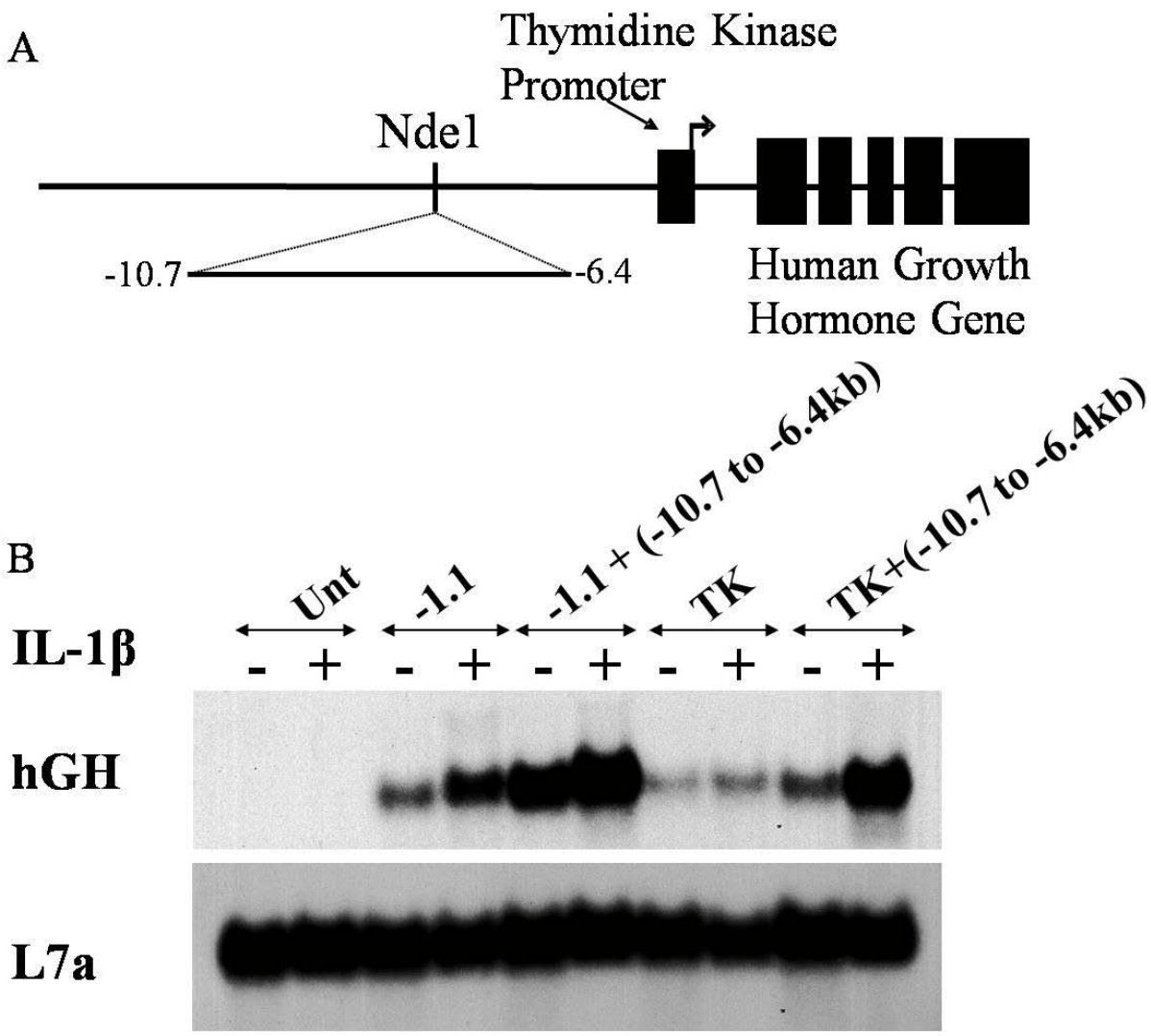

Figure 4-3. HS2 exhibits characteristics of an enhancer: Evaluation of HS2 using a minimal thymidine kinase (TK) heterologous promoter. A) A depiction of the TK promoter fragment driving the expression of human growth hormone (hGH) reporter containing the HS2 fragment -10.7 to -6.4 kb, cloned into the NdeI site. B) HFL-1 cells were transiently transfected with following constructs: 1.1 kb, 1.1 + (-10.7 to -6.4 kb), TK, TK + (-10.7 to -6.4kb). Total RNA was extracted and analyzed by northern blot. The membrane was hybridized with radiolabeled probes for hGH and L7a.

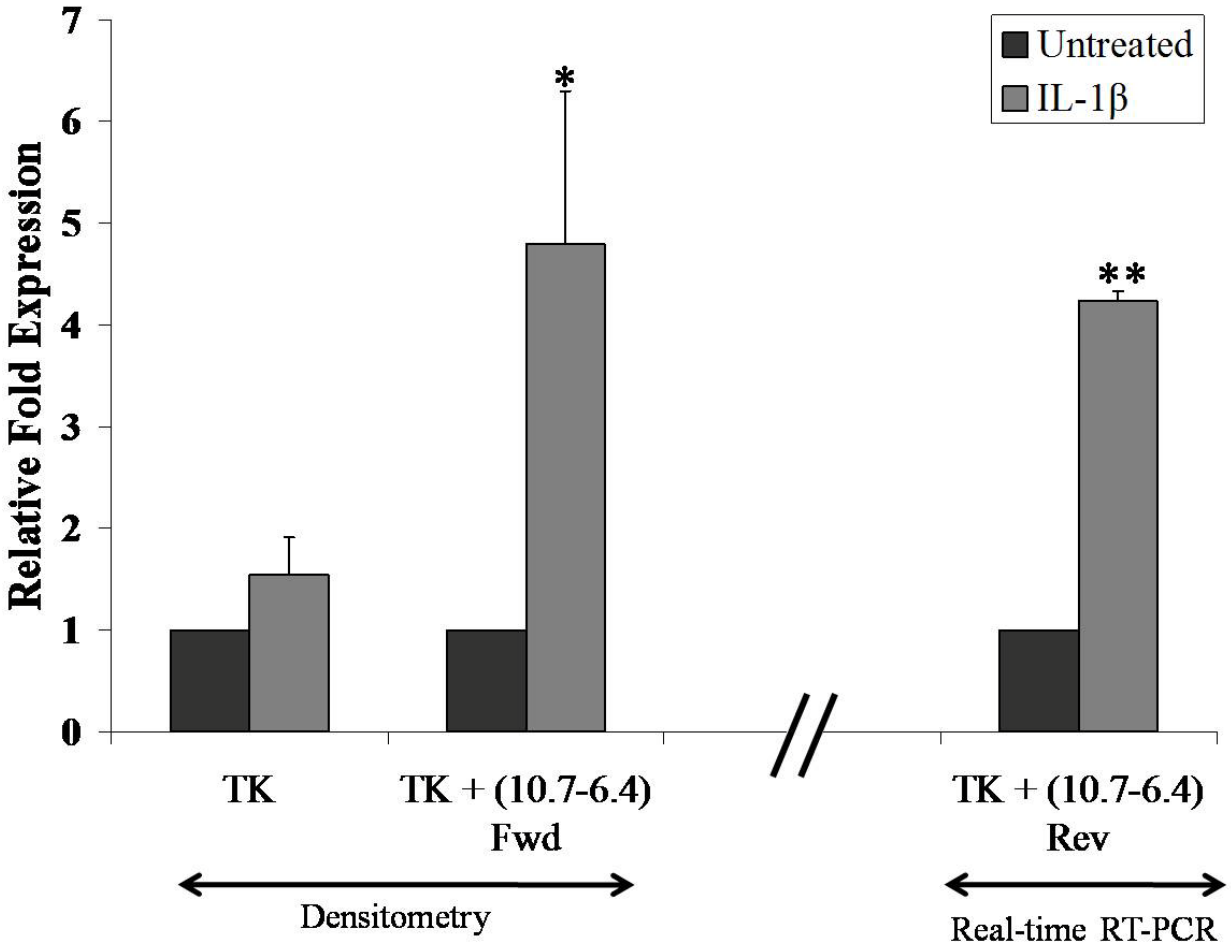

Figure 4-4. HS2 exhibits characteristics of an enhancer: Evaluation of HS2 using a minimal viral thymidine kinase heterologous promoter. The graph depicts densitometry of the wild type TK promoter construct and the HS2 fragment coupled to the TK promoter construct in the forward orientation and real-time RT-PCR analysis of the HS2 fragment cloned in the reverse orientation. The graph is a summary of three independent experiments where data points are represented as mean ± SEM. The asterisk (*) denotes statistical significance with p value $\leq$ 0.05 and (**) denotes statistical significance with p value $\leq$ 0.01 as compared with the untreated sample.

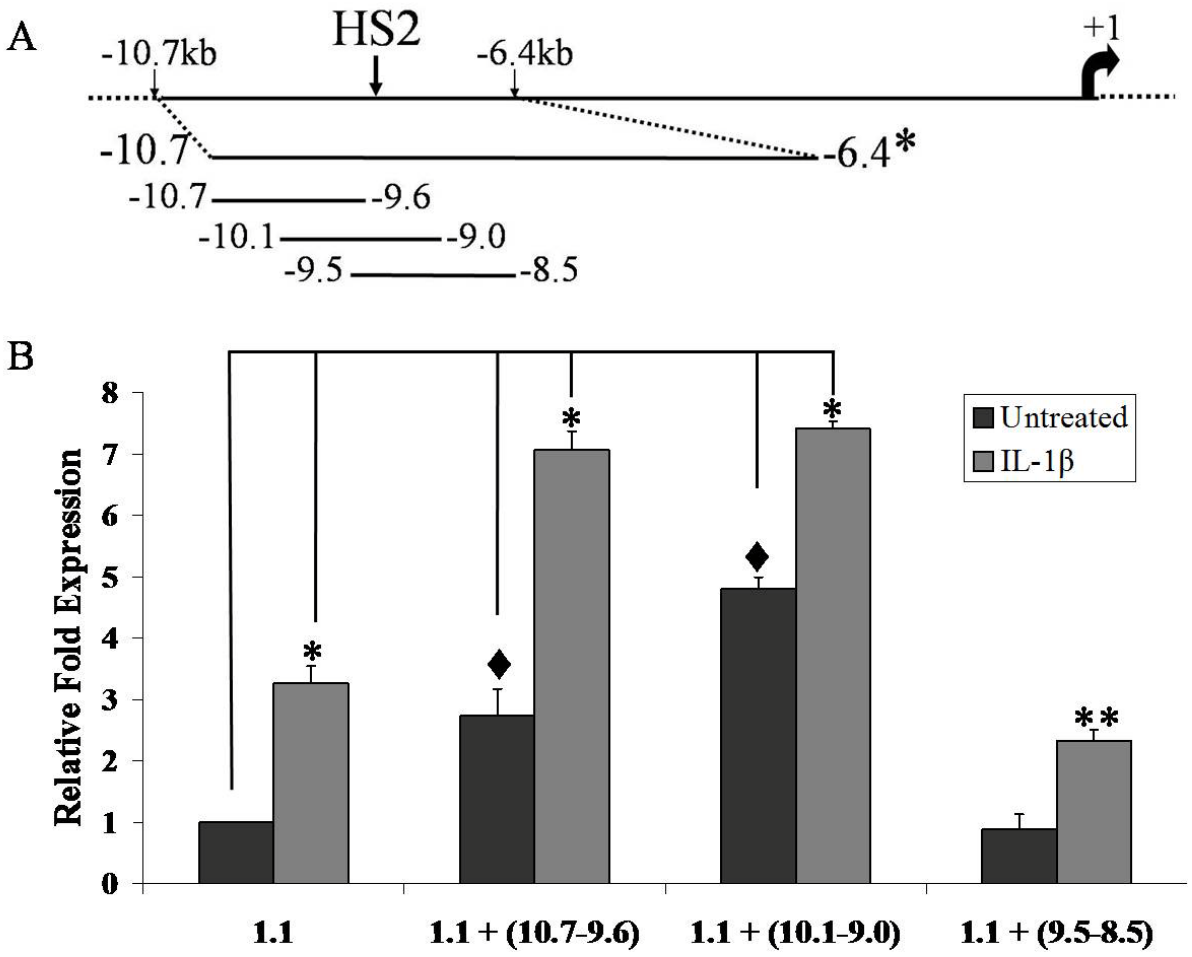

Figure 4-5. Identification of a basal element within HS2. A) A depiction of the HS2 containing fragment -10.7 to -6.4 kb, illustrating the location of a series of 5' overlapping fragments generated by PCR. B) Each fragment was coupled to the -1.1 kb promoter construct, transiently transfected into HFL-1 cells and total RNA was extracted as indicated in the Materials and Methods and analyzed by real-time RT-PCR to detect hGH. The hGH/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of three independent experiments and the data points are represented as mean ± SEM. The diamond (♦) denotes statistical significance with p value $\leq$ 0.05 compared to the untreated wild type promoter. The asterisk (*) denotes statistical significance with p value$\leq$ 0.05 as compared with the untreated wild type promoter. The asterisk (**) denotes statistical significance with p value$\leq$ 0.01 as compared with the untreated sample.

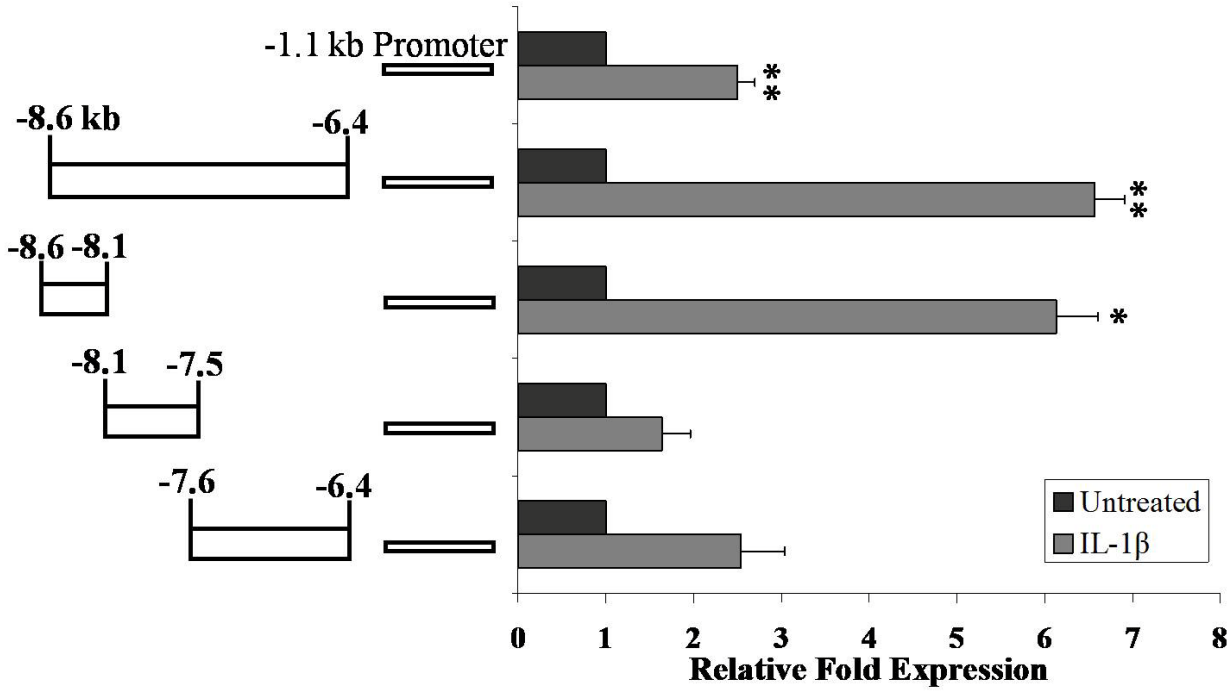

Figure 4-6. Mapping of an inducible element contained within HS2. A series of overlapping internal fragments from the 3'end of the second hypersensitive site (HS2) were generated, -8.6 to -6.4 kb, -8.6 to -8.1 kb, -8.1 to -7.5 kb and -7.6 to -6.4 kb by PCR then coupled to the -1.1 kb promoter construct. Growth home expression was evaluated by transient transfection in HFL-1 cells and real-time RT-PCR analysis. The hGH/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of four independent experiments and the data points are represented as mean ± SEM. The asterisk (*) denotes statistical significance with p value $\leq$ 0.05 and (**) denotes statistical significance with p value $\leq$ 0.01 as compared with the untreated samples.

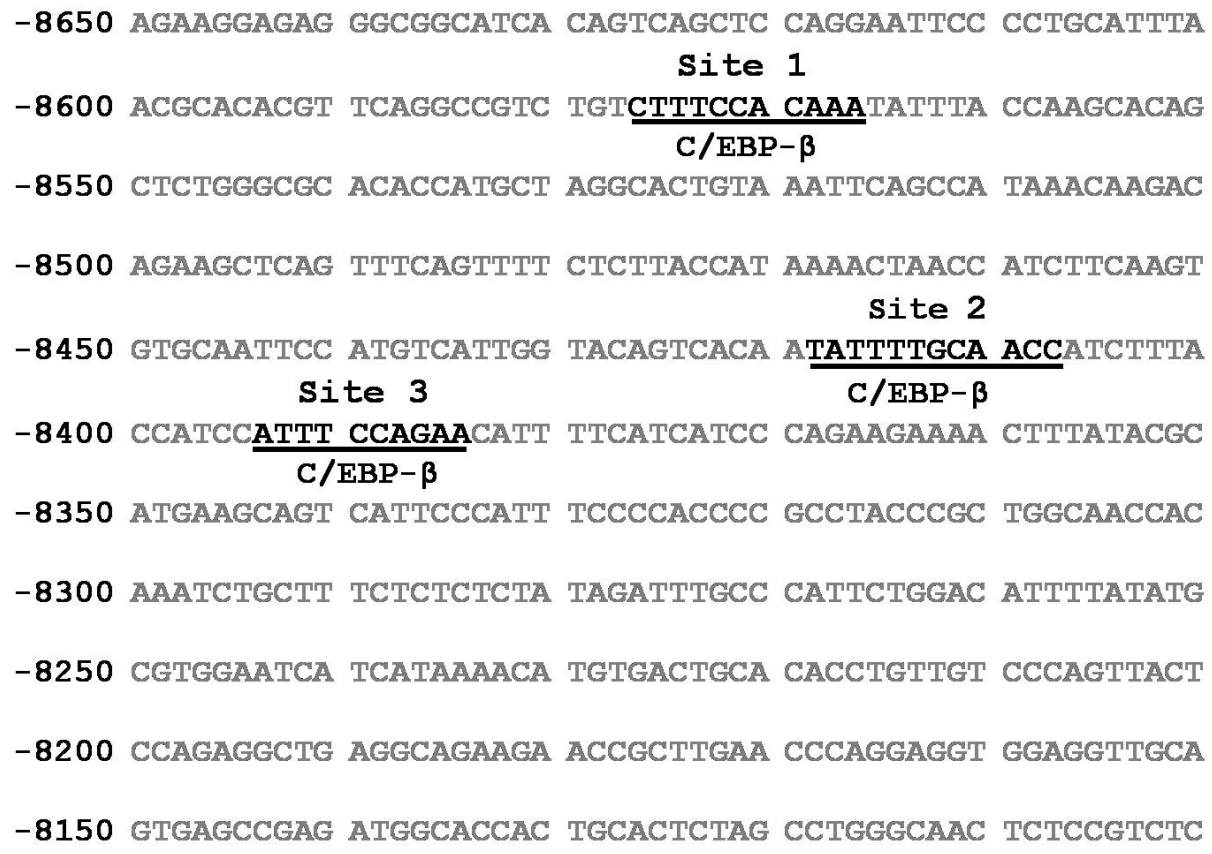

Figure 4-7. Location of three C/EBPβ binding sites in the distal regulatory enhancer element predicted by computer analysis. The (-8.6 to -8.1 kb) fragment sequence was analyzed by TESS – transcription element search software and illustrates the location of the consensus sequence for three C/EBPβ binding sites.

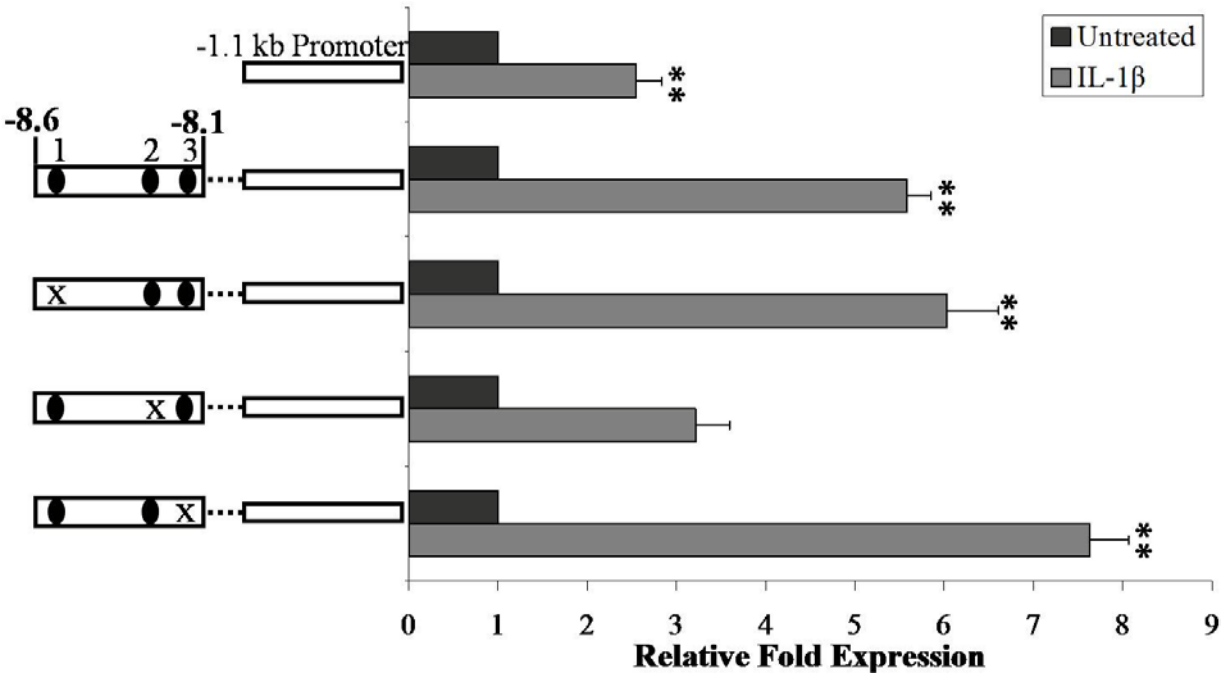

Figure 4-8. Evaluation of single C/EBPβ mutants in the IL-1β induction of mPGES-1. Each of the three C/EBPβ binding sites identified by TESS analysis were mutated by site-directed mutagenesis in the -8.6 to -8.1 kb fragment. These three mutated fragments (-8.6 to -8.1)Δ1, (-8.6 to -8.1)Δ2 and (-8.6 to -8.1)Δ3, coupled to the -1.1 kb promoter construct, were evaluated by transient transfection in HFL-1 cells and real-time RT-PCR analysis to detect hGH expression. The hGH/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of six independent experiments and the data points are represented as mean ± SEM. The asterisk (**) denotes statistical significance with p value≤ 0.01 as compared with the untreated samples. (Note: C/EBPβ sites are represented as filled circles(●) and delete d sites are denoted by an X).

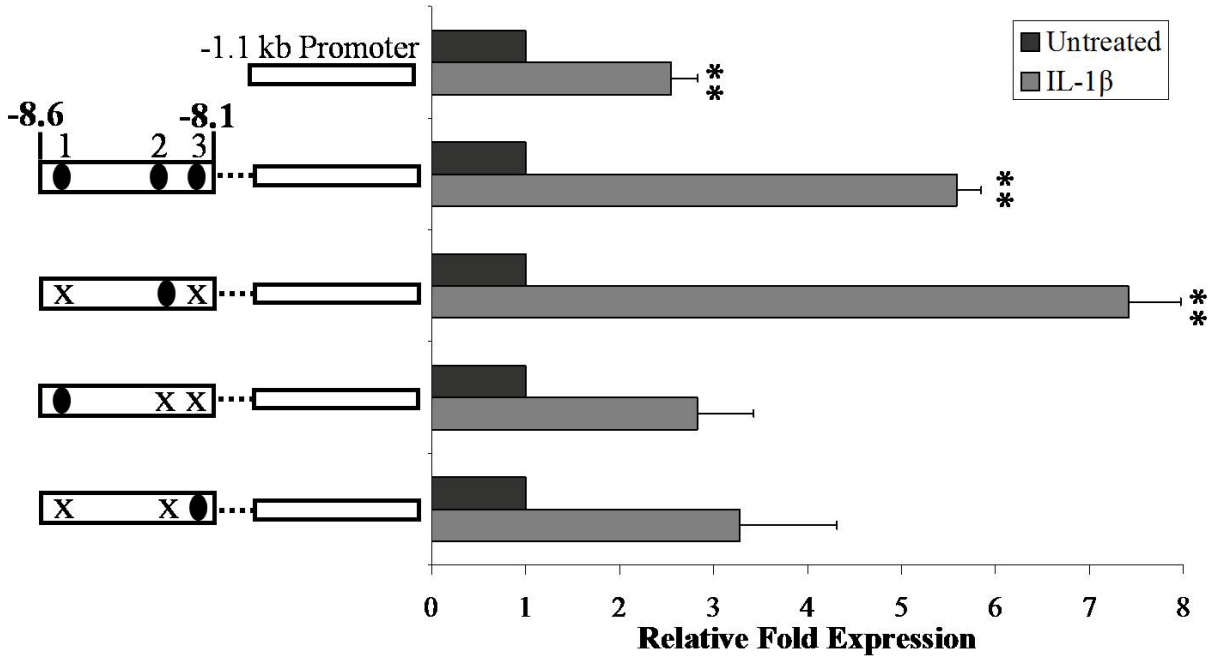

Figure 4-9. Analysis of double C/EBPβ mutants in the IL-1β induction of mPGES-1. A series of double C/EBPβ mutant constructs were generated by site-directed mutagenesis of the -8.6 to -8.1 kb fragment. These three mutated fragments (-8.6 to -8.1)Δ1/3, (-8.6 to -8.1)Δ2/3 and (-8.6 to -8.1)Δ1/2, coupled to the -1.1 kb promoter construct, were compared to the wild type fragment by transient transfection in HFL-1 cells and real-time RT-PCR analysis to detect hGH expression. The hGH/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of six independent experiments and the data points are represented as mean ± SEM. The asterisk (**) denotes statistical significance with p value ≤ 0.01 as compared with the untreated samples. (Note: C/EBPβ sites are represented as filled circles (●) and deleted sites are denoted by an X).

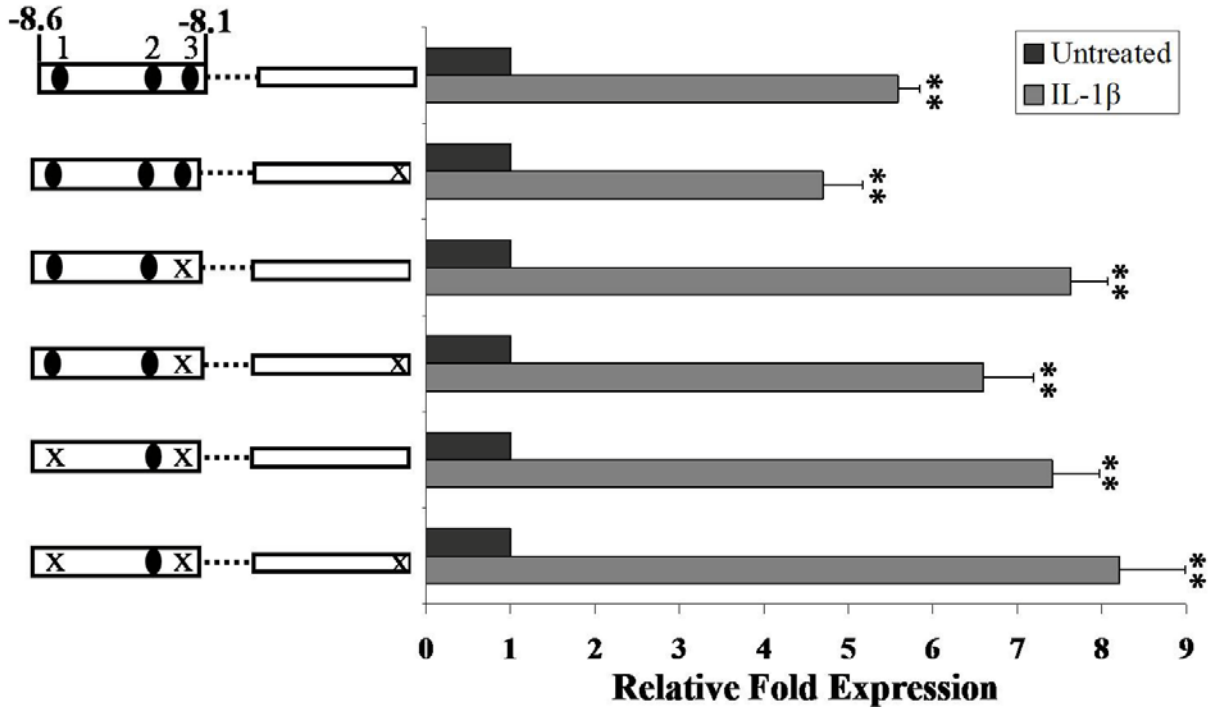

Figure 4-10. Evaluation of the C/EBPβ sites in constructs lacking the Egr-1 binding site. The following fragments (8.6 to 8.1), (8.6 to 8.1)Δ3, (8.6 to 8.1)Δ1/3, coupled to the ΔEgr-1 promoter construct, were compared to the wild type fragments, (8.6 to 8.1), (8.6 to 8.1)Δ3, (8.6 to 8.1)Δ1/3, following transient transfection in HFL-1 cells and real-time RT-PCR analysis to detect hGH expression. The hGH/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of three independent experiments and the data points are represented as mean ± SEM. The asterisk (**) indicates statistical significance with p value ≤ 0.01 relative to untreated samples. (Note: C/EBPβ sites are represented as filled circles() and deleted sites are denoted by an X).

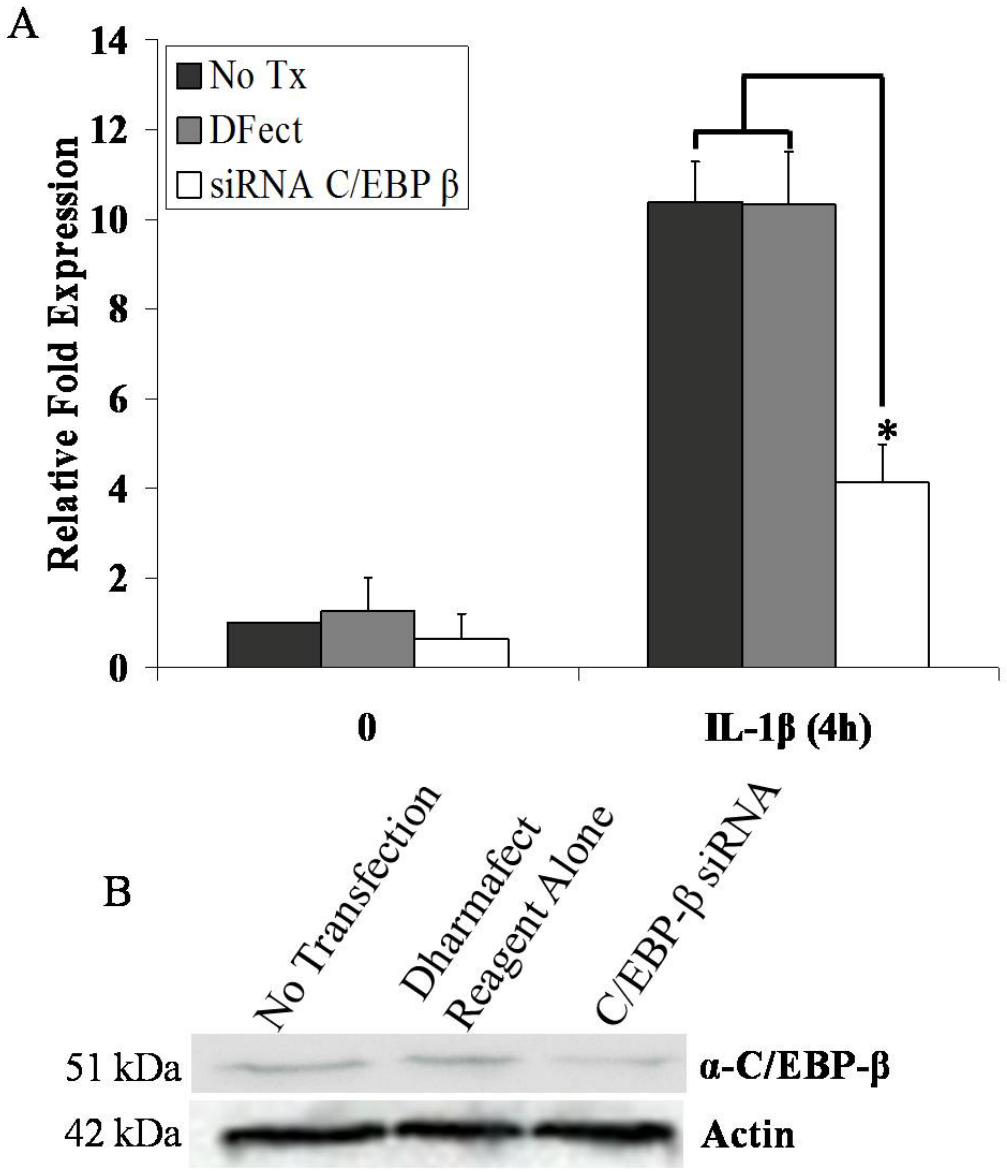

Figure 4-11. Targeted deletion of C/EBPβ by siRNA in human lung fibroblasts. A) HFL-1 cells, were mock-transfected (vehicle alone) or transfected with a Dharmafect siRNA specifically targeting human C/EBPβ, with or without 4h IL-1β treatment. Total RNA was extracted and subjected to real-time RT-PCR analysis to detect either mPGES-1 or cyclophilin A mRNA. The mPGES-1/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of three independent experiments, where the data points are represented as mean ± SEM. The asterisk (*) indicates statistical significance with p value≤ 0.05 as compared with the control sample. B) Immunoblot analysis of C/EBPβ knockdown in HFL-1 cells using a rabbit polyclonal antibody against C/EBPβ. A mouse monoclonal antibody against actin was used as a loading control.

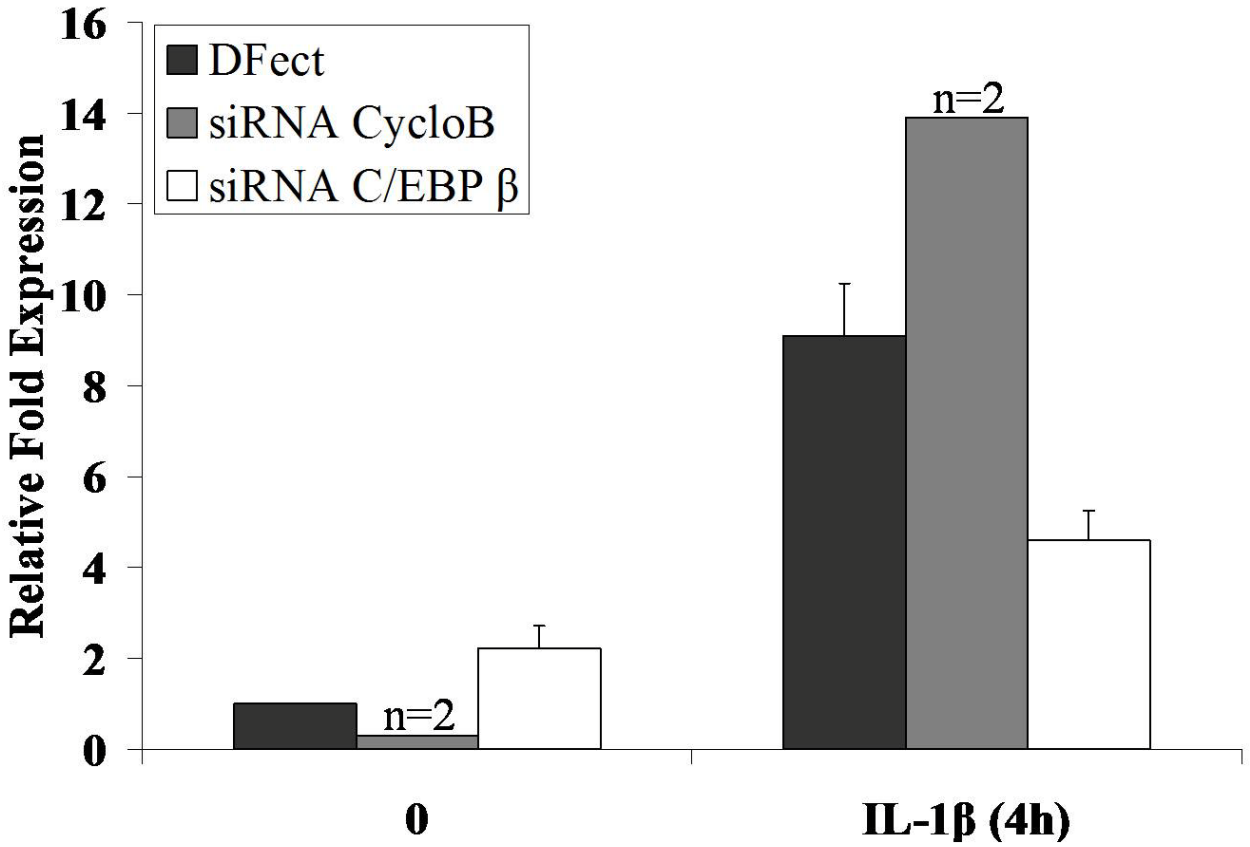

Figure 4-12. Targeted deletion of C/EBPβ by siRNA in rat lung epithelial cells. Rat pulmonary epithelial cells, L2, were mock-transfected (vehicle alone) or transfected with a Dharmafect siRNA specifically targeting either rat cyclophilin B or C/EBPβ, respectively, with or without 4h IL-1β treatment. Total RNA was extracted and subjected to real-time RT-PCR analysis to detect either mPGES-1 or cyclophilin A mRNA. The mPGES-1/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of three independent experiments, where the data points are represented as mean ± SEM.

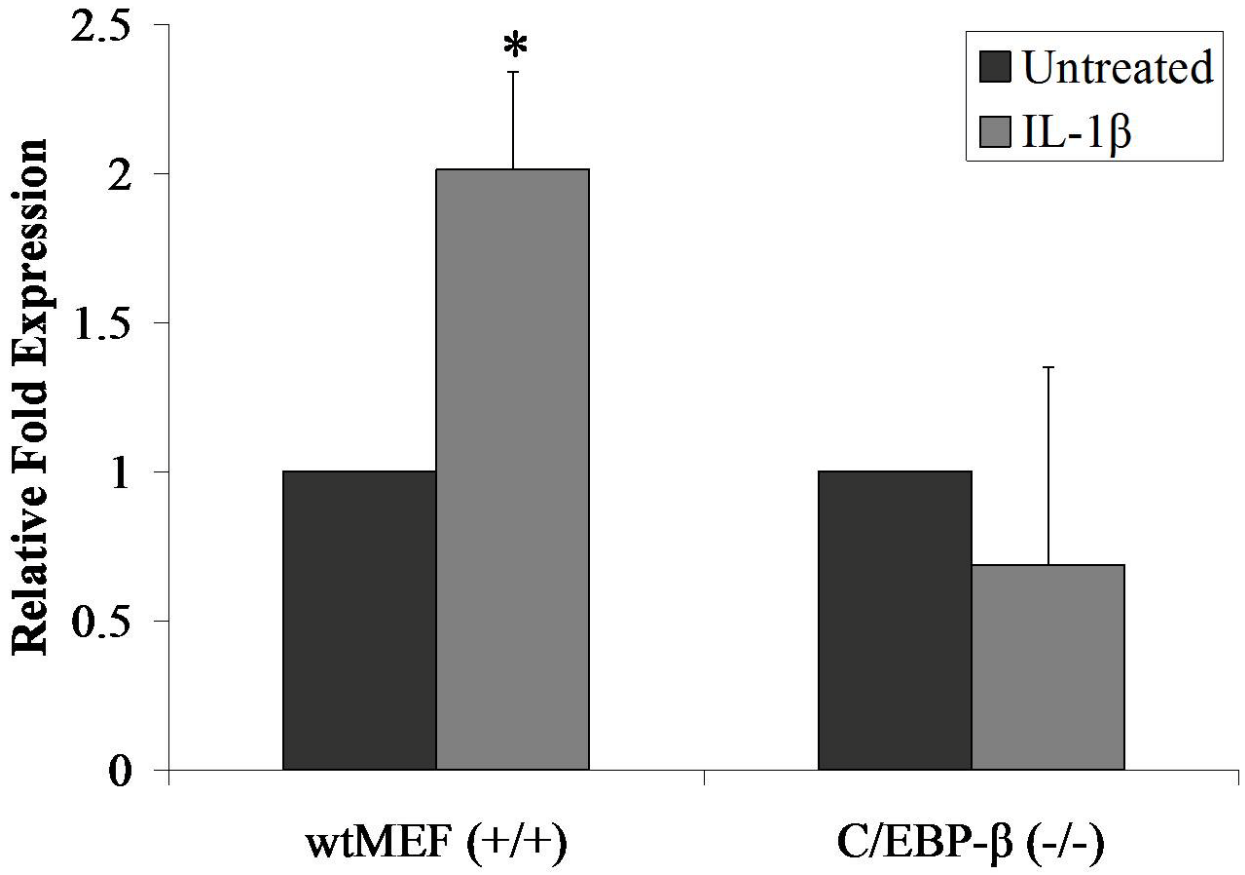

Figure 4-13. Evaluation of mPGES-1 expression in C/EBPβ-deficient mouse embryonic fibroblast (MEF) cells. Wild type (+/+) and C/EBPβ (-/-) MEFs were untreated or stimulated with 2ng/mL IL-1β for 8h. Total RNA was extracted and subjected to real-time RT-PCR analysis to detect mPGES-1 or cyclophilin A mRNA. The mPGES-1/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of four independent experiments, where the data points are represented as mean ± SEM. The asterisk (*) indicates statistical significance with p value≤ 0.05 as compared with the untreated sample.

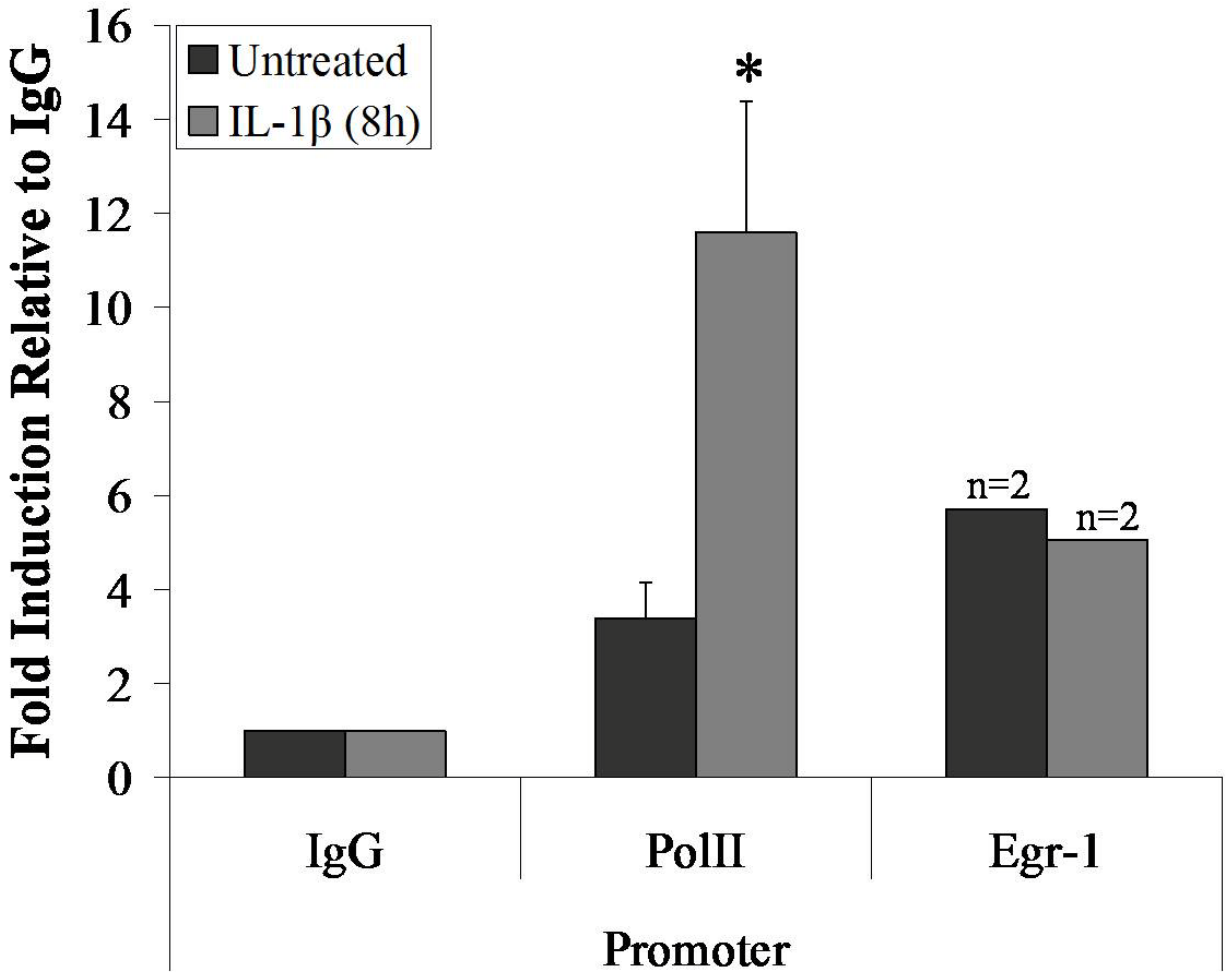

Figure 4-14. Chromatin immunoprecipitation analysis of Egr-1 and RNA Polymerase II binding. HFL-1 cells were stimulated with 2ng/mL IL-1β for 0 and 8 h, and subjected to a ChIP assay as described in the Experimental Procedures with control IgG, RNA Polymerase II or Egr-1 specific antibodies. All values are graphed as a fraction of input relative to IgG ± SEM. The asterisk (*) indicates statistical significance with p value $\leq$ 0.05 as compared with the untreated samples.

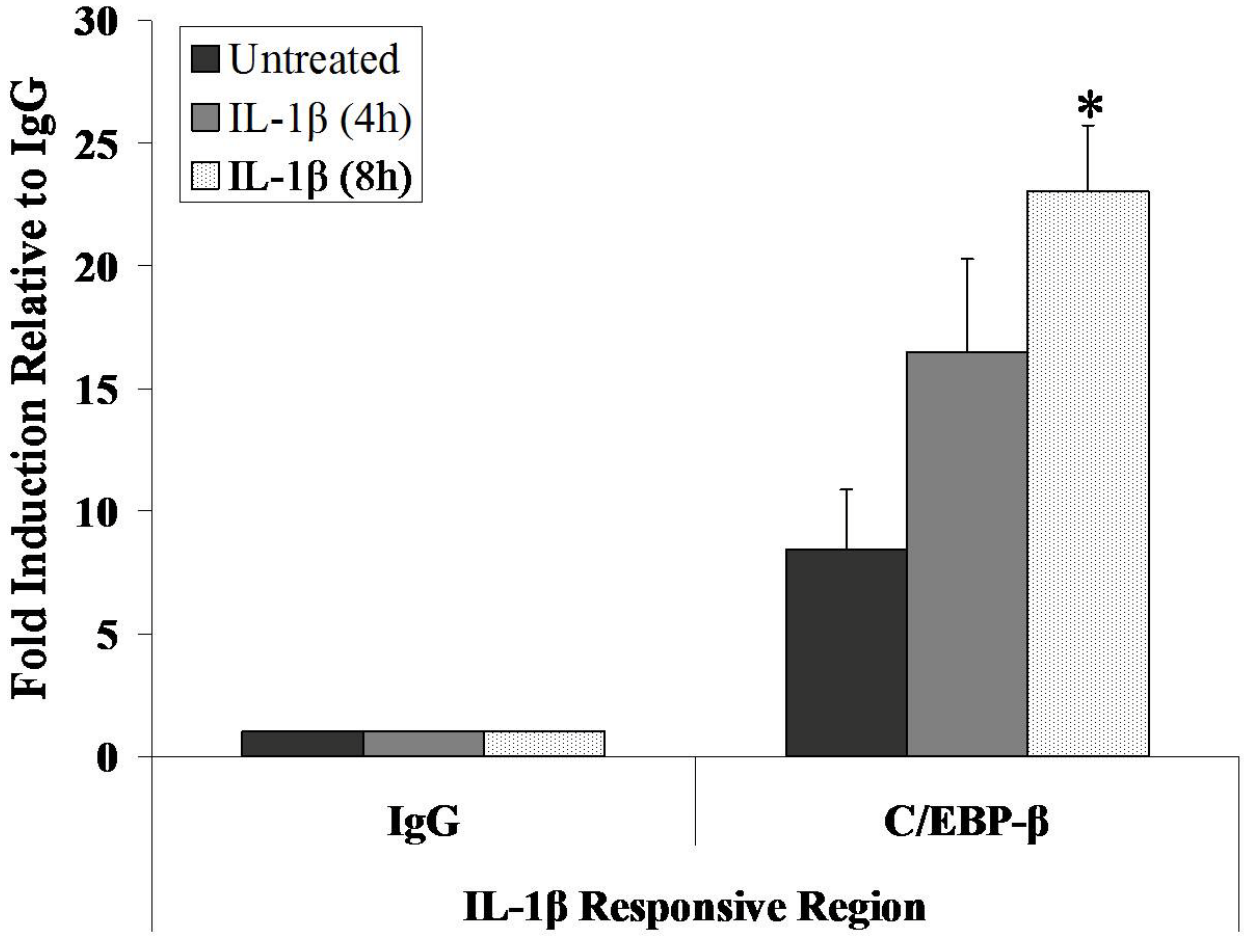

Figure 4-15. Chromatin immunoprecipitation analysis of C/EBPβ binding. ChIP analysis of HFL-1 cells stimulated with IL-1β for 0, 4 and 8 h using control IgG and C/EBPβ specific antibodies. All values are graphed as a fraction of input relative to IgG ± SEM. The asterisk (*) indicates statistical significance with p value ≤ 0.05 as compared with the untreated samples.

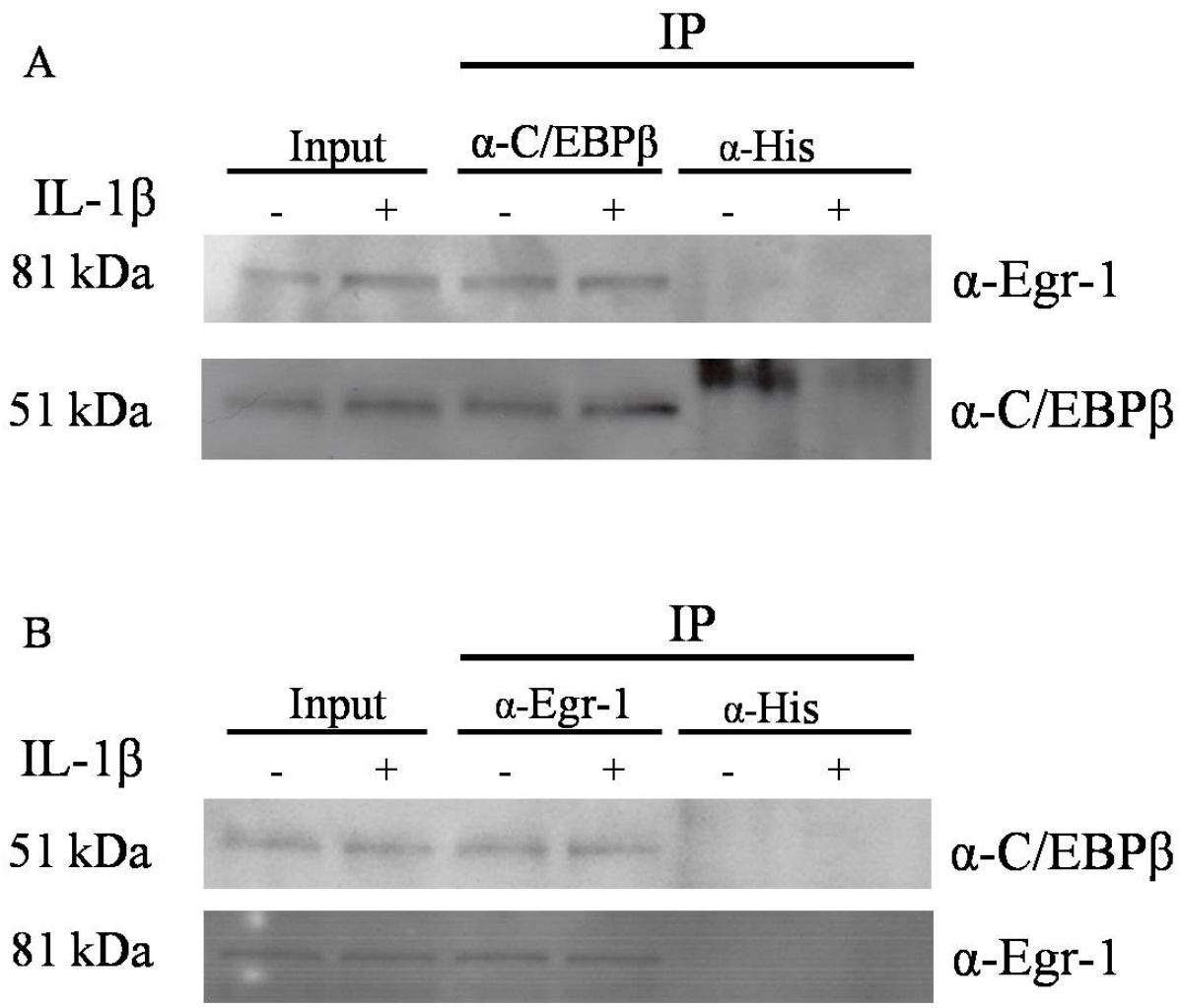

Figure 4-16. Co-immunoprecipitation analysis of Egr-1 and C/EBPβ binding. A) HFL-1 cells were stimulated with IL-1β for 8 h then subjected to co-immunoprecipitation with a mouse monoclonal antibody against C/EBPβ followed by immunoblot analysis with a rabbit polyclonal antibody against Egr-1. An antibody against a polyhistidine peptide was used a control. The membrane was reprobed with a rabbit polyclonal antibody against C/EBPβ to confirm its expression. B) Co- immunoprecipitation analysis in IL-1β stimulated HFL-1 cells with a mouse monoclonal antibody against Egr-1 followed by immunoblot analysis with a rabbit polyclonal antibody against C/EBPβ. An antibody against a polyhistidine peptide was used as a control. The membrane was also reprobed with a rabbit polyclonal antibody against Egr-1 to confirm its expression.

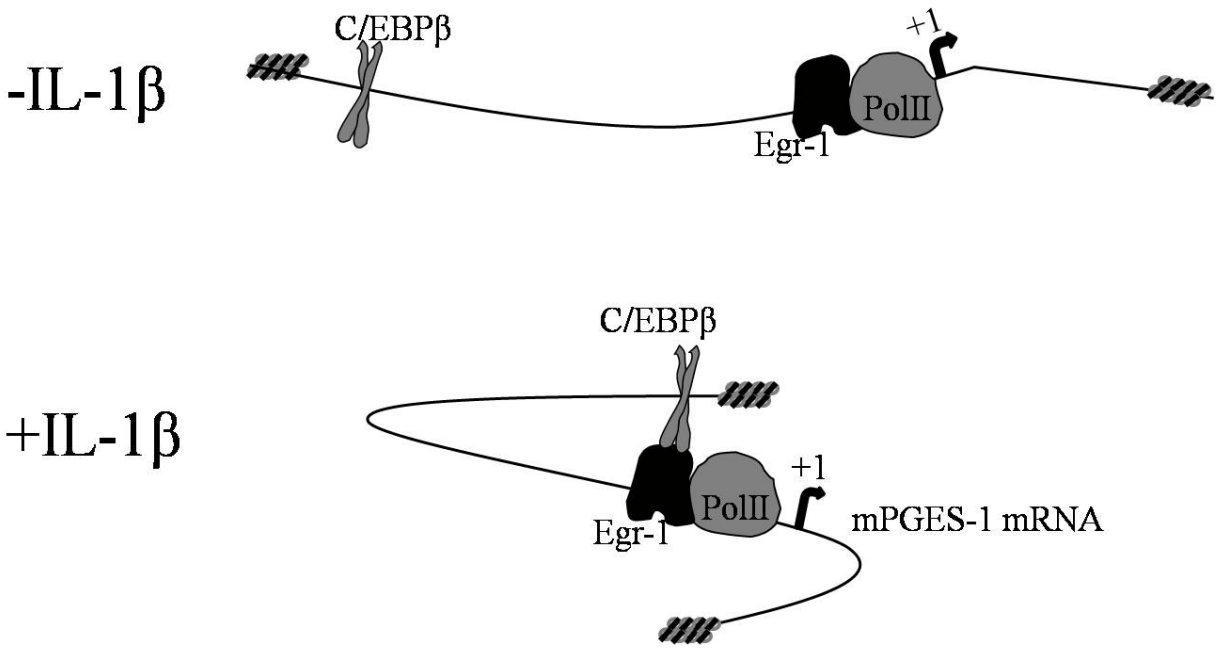

Figure 4-17. Model of the functional role played by C/EBPβ and Egr-1 in activating the IL-1β induction of mPGES-1 gene expression. In the absence of stimulus, Egr-1 and RNA Polymerase II bind to the proximal promoter while C/EBPβ binds to the distal enhancer element leading to basal expression of mPGES-1. In the presence of IL-1β, there is increased RNA Polymerase II binding at the proximal promoter region, perhaps stabilized by the presence of Egr-1 and there is an increase in C/EBPβ binding to the distal region. There is potential cross-talk between Egr-1 and C/EBPβ leading to the up-regulation of mPGES-1 expression.

CHAPTER 5
P38MAPK, CYTOSOLIC PHOSPHOLIPASE A2 ALPHA AND 15-LIPOXYGENASE (15-LOX) ACTIVITIES ARE REQUIRED FOR TRANSCRIPTIONAL INDUCTION OF CYTOSOLIC PHOSPHOLIPASE A2 ALPHA BY INTERLEUKIN-1BETA: A FEED-FORWARD MECHANISM

Introduction

Cytosolic Phospholipase $A_2\alpha$ ($cPLA_2\alpha$) Activation is Dependent on Phosphorylation and Intracellular Calcium Levels

As the principal enzyme involved in liberating arachidonic acid from membrane phospholipids, $cPLA_2\alpha$ activation and regulation are considered one of the rate-limiting steps in arachidonic acid metabolism (91,260). $cPLA_2\alpha$ contains a C2 domain which has been shown to regulate the binding of intracellular Ca^{2+} and the translocation of $cPLA_2\alpha$ from the cytosol to the perinuclear membrane; bringing the enzyme in close contact with its substrate and downstream enzymes involved in arachidonic acid metabolism (109,261,262). $cPLA_2\alpha$ also contains two catalytic domains interspaced with isoform specific sequences (261). Three serine residues, Ser505, Ser515 and Ser727, located in the linker sequences surrounding the second catalytic domain, have been implicated in the regulation of $cPLA_2\alpha$ enzymatic activity (104,113). Phosphorylation of Ser505, Ser515 and Ser727 by mitogen activated protein kinase (MAPK) (104), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (263) and the MAPK-interacting kinase (MNK1) (108) is known to increase $cPLA_2\alpha$ enzymatic activity. Alternatively, a number of studies including a 1995 study by Schievella et al. (112) have shown that deletion of the C2 domain abrogates $cPLA_2\alpha$ translocation to the perinuclear membrane while mutation of Ser505 had no effect on $cPLA_2\alpha$ translocation (109,112). Further, the role of phosphorylated Ser505 in regulating $cPLA_2\alpha$ enzymatic activity was evaluated by over expression of a mutant Ser505, S505A; the results illustrated a reduction in agonist-induced arachidonic acid release in S505A mutant cells compared to wild type $cPLA_2\alpha$ over-expressing cells (104). Overall both

intracellular Ca^{2+} levels and phosphorylation of serine residues in the linker regions of cPLA$_2$α by MAPK and other kinases have been shown to be involved in regulating of cPLA$_2$α activity.

A number of studies have illustrated that IL-1β stimulates the rapid induction of cPLA$_2$α phosphorylation, with a concomitant increase in cPLA$_2$α enzymatic activity within 15 – 30 min of treatment (264,265), while cPLA$_2$α mRNA expression occurs a few hours after IL-1β induction (96,266,267). Our lab previously showed that cytokine-induced cPLA$_2$α gene expression was a consequence of *de novo* transcription (96). Furthermore cPLA$_2$α gene expression is known to be inhibited by glucocorticoid treatment (267-269). While a handful of studies have evaluated the proximal promoter region of cPLA$_2$α and identified a number of putative transcription factor binding sites involved in basal expression of cPLA$_2$α (96,270,271), the direct mechanism involved in the cytokine-mediated induction of cPLA$_2$α is still unknown.

The information presented thus far illustrates the involvement of kinase pathways in regulating cPLA$_2$α enzymatic activity and highlights the need for studies evaluating the mechanisms underlying the cytokine-mediated induction of cPLA$_2$α gene expression. Therefore, the work presented in this chapter attempted to elucidate the signaling mechanisms involved in the cytokine-mediated induction of cPLA$_2$α gene expression. Kinase pathways involved in mediating enzymatic activation and cPLA$_2$α gene expression will be evaluated, together with the activities of downstream arachidonic acid metabolites as part of a feed forward mechanism.

It should be noted that the results in this chapter were performed in conjunction with Dr. J.D. Herlihy and Ms. Molly Strickland. Some of the studies have been previously presented in Dr. Herlihy's dissertation; however the organization, compilation of all the data, data analysis and a number of additional studies were conducted by myself. A manuscript describing these

studies with myself as first author is under review with the ***Journal of Biological Chemistry*** and a revised manuscript will be submitted after the completion of my dissertation.

Results

IL-1β Induces Cytosolic $PLA_2\alpha$ Phosphorylation via the Action of P38MAPK

Previous studies have identified the involvement of MAPK in regulating $cPLA_2\alpha$ enzymatic activity; MAPK mediates the phosphorylation of $cPLA_2\alpha$ leading to a rapid induction of its enzymatic activity (104). Alternatively, this rapid induction of $cPLA_2\alpha$ enzymatic activity, which occurs on the minute time scale, also leads to a rapid increase in arachidonic acid levels (272). Furthermore, the pro-inflammatory stimuli, IL-1β, TNFα or LPS, are known to induce $cPLA_2\alpha$ mRNA and protein expression which occurs ~1 to 2 h after stimulation (96,99,273,274). Therefore, in an attempt to explore whether signaling by the pro-inflammatory cytokine, IL-1β, involves phosphorylation of $cPLA_2\alpha$, HFL-1 cells were treated with IL-1β in the absence or presence of a known p38MAPK inhibitor, SB203580. Total protein was isolated and evaluated by immunoblot analysis with a phospho-specific antibody recognizing Ser505 of $cPLA_2\alpha$. The results in Figure 5-1(A) illustrate that IL-1β caused a significant increase in $cPLA_2\alpha$ phosphorylation within 10 min and reaching maximal levels by 1 h. Also, treatment with the p38MAPK inhibitor, SB203580, blocked the IL-1β-dependent phosphorylation of $cPLA_2\alpha$, suggesting a role for p38MAPK in the phosphorylation and thus rapid activation of $cPLA_2\alpha$ enzymatic activity. The chart in Figure 5-1(B) illustrates quantitative statistical analysis of three independent experiments.

P38MAPK Mediates Cytosolic $PLA_2\alpha$ Gene Expression in an IL-1β-dependent Manner

There have been conflicting reports as to the involvement of other kinases such as MNK-1 (108), ERK1/2 (275) or CaMKII (105,276,277) in the phosphorylation and rapid activation of $cPLA_2\alpha$ enzymatic activity. Also, previous work by our lab demonstrated that $cPLA_2\alpha$ gene

expression is induced at the transcriptional level by IL-1β and TNFα (96), whereby treatment with either pro-inflammatory cytokine induced $cPLA_2\alpha$ mRNA and protein expression in a time-dependent manner. Therefore, to correlate the rapid phosphorylation event with transcriptional induction of $cPLA_2\alpha$ and illustrate the specificity of p38MAPK in the activation of $cPLA_2\alpha$, induction of $cPLA_2\alpha$ gene expression was evaluated. HFL-1 cells were treated with inhibitors of p38MAPK (SB203580 and SB202190), ERK1/2 (PD98059) and JNK (SP600125) in the absence or presence of IL-1β for 8 h. Total RNA was isolated and analyzed by northern blot. The results in Figure 5-2(A) illustrate that both p38MAPK inhibitors effectively blocked the IL-1β-dependent induction of $cPLA_2\alpha$ gene expression. Further, $cPLA_2\alpha$ protein expression was evaluated in HFL-1 cells following treatment with the JNK inhibitor (SP600125) and the p38MAPK inhibitor (SB203580). The data in Figure 5-2(B) demonstrates that treatment with the p38MAPK inhibitor caused a reduction in $cPLA_2\alpha$ protein levels.

To systematically demonstrate the specific involvement of p38MAPK in the transcriptional induction of $cPLA_2\alpha$, $cPLA_2\alpha$ gene expression was evaluated by northern blot following treatment with p38MAPK inhibitors. HFL-1 cells were exposed to varied concentrations of both p38MAPK inhibitors, SB203580 or SB202190, in the absence or presence of IL-1β for 8 h. Figure 5-3(A and B) illustrate that both p38MAPK inhibitors blocked the IL-1β-dependent induction of $cPLA_2\alpha$ gene expression in a dose dependent manner. The chart in Figure 5-4 is densitometric analysis of three independent experiments for SB203580.

There are four known p38MAPK isoforms, p38α, p38β, p38γ and p38δ. Of the four, two isoforms, p38α and p38β have been widely studied and are known to be involved in the regulation and activation of many different genes involved in numerous cellular and biological processes (278-280). As a specific verification of the involvement of p38MAPK, mouse

embryonic fibroblasts (MEF), wild type and p38α or p38β deficient were utilized to evaluate $cPLA_2\alpha$ gene expression. MEF cells were stimulated with IL-1β for 8 h, total RNA was extracted and analyzed by real-time RT-PCR which mouse-specific $cPLA_2\alpha$ primers. The data in Figure 5-5 illustrates that $cPLA_2\alpha$ gene expression is induced ~1.5 fold in both the p38α and p38β wild-type MEFs, but in the absence of p38α or p38β, IL-1β is not able to induce $cPLA_2\alpha$ expression.

Together, the data in Figure 5-1 to Figure 5-5 highlight for the first time, the specific involvement of p38MAPK in both the rapid induction of $cPLA_2\alpha$ enzymatic activity within 30 min to 1 h and the IL-1β-dependent induction of $cPLA_2\alpha$ gene expression over a longer time period. These results also suggest a potential role for other kinases, that are activated by p38 or which activate p38, in mediating the IL-1β-dependent induction of $cPLA_2\alpha$ expression. In the next sections, individual kinases known to be upstream and downstream of p38 will be evaluated for their involvement in the IL-1β induction of $cPLA_2\alpha$ gene expression.

Phosphorylation of MKK3/MKK6 is Induced by IL-1β

For years, numerous studies have researched and elucidated the intracellular signaling cascade for MAPK activation (130,281,282). It is known that activation of p38MAPK is mediated by the actions of the dual upstream kinases MKK3 and MKK6 (283,284). Therefore, in this study MKK3/MKK6 activation by IL-1β will be evaluated. HFL-1 cells were stimulated with IL-1β over the course of 1 h, protein was isolated and analyzed by immunoblotting with a dual phospho-specific antibody recognizing Ser189 (MKK3) and Ser207 (MKK6). The results in Figure 5-6(A) illustrate that in a time dependent manner, IL-1β caused a significant increase in MKK3/MKK6 phosphorylation. A graph depicting densitometry of three independent experiments is also shown in Figure 5-6(B).

To further demonstrate the role of MKK3/MKK6 in p38MAPK activation and subsequent activation of $cPLA_2\alpha$ gene expression, mouse embryonic fibroblasts, wild type and MKK3/MKK6-deficient, were evaluated for $cPLA_2\alpha$ gene expression. Wild type and deficient MEF cells were stimulated with IL-1β for 8 h, total RNA was extracted and analyzed by real-time RT-PCR. The data in Figure 5-7 demonstrates that IL-1β significantly induced $cPLA_2\alpha$ mRNA levels approximately 1.9 fold in the wild type MEFs but not in the MKK3/MKK6-deficient MEF. These results illustrate that IL-1β is capable of inducing phosphorylation of MKK3/MKK6, a kinase upstream of p38MAPK, which is directly involved in the phosphorylation and subsequent activation of p38MAPK. Further, this data demonstrates the involvement of MKK3/MKK6 in the IL-1β mediated induction of $cPLA_2\alpha$ gene expression.

Phosphorylation of MSK-1 is Induced by IL-1β

Within the MAPK signaling cascade there is a kinase, MSK1, which is believed to be downstream of p38 (280). A nuclear kinase, MSK-1 is known to be phosphorylated and subsequently activated by p38MAPK (285-287). Studies have shown that activation of MSK-1 is involved in the regulation and activation of various transcription factors such as nuclear factor-kappa B (288), cAMP-response element-binding protein (287,289) and the chromatin remodeling proteins histone H3 and HMG-14 (290). Therefore, to determine whether treatment with IL-1β could induce phosphorylation of MSK-1 and whether p38MAPK inhibition has any effect on this phosphorylation event, HFL-1 cells were treated with IL-1β in the absence or presence of the p38MAPK inhibitor, SB203580. As shown in Figure 5-8(A) IL-1β stimulates the phosphorylation of MSK-1 within 10 minutes of treatment, while co-treatment with SB203580 blocks the phosphorylation event.

The data presented thus far illustrates, that IL-1β induces the phosphorylation of MKK3/MKK6. This kinase then goes on to activate p38MAPK which leads to the

phosphorylation and rapid activation of cPLA$_2$α enzymatic activity and gene expression. Within this cascade, p38MAPK is involved in the phosphorylation of MSK-1, demonstrated in Figure 5-8(A and B) where IL-1β caused the rapid phosphorylation of MSK-1, while the p38MAPK inhibitor SB203580 blocked the IL-1β-dependent induction. It should be noted that attempts to procure MSK-1 deficient fibroblasts were not successful. In the next section, the involvement of downstream metabolites of arachidonic acid will be evaluated for their role in the IL-1β-mediated induction of cPLA$_2$α gene expression.

Inhibition of Cytosolic PLA$_2$α Enzymatic Activity Blocks the IL-1β Induction of Cytosolic PLA$_2$α Gene Expression: A Feed Forward Mechanism

It has been postulated that rapid activation of cPLA$_2$α enzymatic activity may lead to an increase in cPLA$_2$α gene expression and increased levels of free arachidonic acid (272). In 1994, Bartoli et al. (291) demonstrated that specific inhibition of cPLA$_2$α enzymatic activation blocked thrombin-induced release of arachidonic acid, through tight association of the specific cPLA$_2$α inhibitor, trifluoromethyl ketone (AACOCF$_3$) with cPLA$_2$α. We hypothesized that the rapid induction of cPLA$_2$α enzymatic activity may play a role in the transcriptional induction of cPLA$_2$α gene expression through a feed forward mechanism.

HFL-1 cells were treated with the cPLA$_2$α inhibitor, arachidonyl trifluoromethyl ketone (AACOCF$_3$) in the absence or presence of IL-1β for 8 h. Figure 5-9(A) illustrates northern analysis of cPLA$_2$α mRNA expression and reveals that AACOCF$_3$ blocks the IL-1β mediated induction of cPLA$_2$α gene expression in a dose dependent manner. The graph in Figure 5-9(B) represents densitometry of three independent experiments. Utilizing another specific cPLA$_2$α inhibitor, pyrrolidine, the data in Figure 5-10(A) further illustrates the dose dependent decrease in the IL-1β induction of cPLA$_2$α gene expression following treatment with pyrrolidine. The chart in Figure 5-10(B) shows an average of two independent experiments. Together these

results demonstrate involvement of arachidonic acid and possibly its downstream metabolites as part of a feed forward mechanism in the IL-1β induction of $cPLA_2\alpha$.

The Lipoxygenase Pathway but not Cyclooxygenase Pathway is Necessary for Cytosolic $PLA_2\alpha$ Expression

It is widely accepted that $cPLA_2\alpha$ liberates arachidonic acid from membrane phospholipids for metabolism and downstream eicosanoid signaling as illustrated in Figure 1-1. In the next series of experiments the arachidonic acid metabolites were evaluated to determine their involvement in regulating $cPLA_2\alpha$ gene expression. To evaluate the involvement of the cyclooxygenase (COX) pathway, HFL-1 cells were treated with a non-selective COX inhibitor, indomethacin, in the absence or presence of IL-1β for 8 h. Total RNA was isolated and analyzed by northern blot, and the results in Figure 5-11 revealed that treatment with indomethacin had no effect on the induction of $cPLA_2\alpha$ gene expression by IL-1β. As a consequence of these results, the involvement of the lipoxygenase pathway was then evaluated. HFL-1 cells were treated with the non-selective LOX inhibitor, nordihydroguaiaretic acid (NDGA), a polyphenol derivative (292), in the absence or presence of IL-1β for 8 h. The data in Figure 5-12(A) illustrates that treatment with NDGA blocks the induction of $cPLA_2\alpha$ gene expression by IL-1β. Densitometry of three independent experiments is shown in Figure 5-12(B).

Since NDGA inhibits 5-, 12- and 15-LOX, it was important to determine which of the three LOX enzymes was required for the IL-1β induction of $cPLA_2\alpha$ gene expression. The lipoxygenase enzymes are involved in converting arachidonic acid to leukotrienes (5-LOX mediated reaction) and 15-HETEs (15-LOX mediated reaction) (67,132,136). 5-lipoxygenase activating protein (FLAP) is known to regulate 5-LOX activation and the inhibitor, MK-886 is known to specifically inhibit FLAP activity (293,294). Therefore, HFL-1 cells were treated with the 5-LOX inhibitor, MK886, in the absence or presence of IL-1β, total RNA was isolated and

analyzed by northern blot. The results in Figure 5-13 indicate that 5-LOX activity is not involved in the IL-1β-dependent induction of $cPLA_2\alpha$ gene expression.

The previous results have ruled out the involvement of 5-LOX in the IL-1β induction of $cPLA_2\alpha$ gene expression. Therefore to determine the specific involvement of 12-LOX or 15-LOX, a pharmacological inhibitor of either 12-LOX or 15-LOX was used. Previously HFL-1 cells were treated with baicalein, a known inhibitor of 12-LOX (295), and the results of that experiment indicated that 12-LOX did not play a role in the IL-1β induction (data not shown). To illustrate the specific involvement of 15-LOX, HFL-1 cells were treated with a potential 15-LOX inhibitor, luteolin, a plant flavonoid, in the absence or presence of IL-1β for 8 h. Total RNA was isolated and analyzed by northern blot. The results in Figure 5-14(A) indicate that luteolin reduced both the basal and induced expression of $cPLA_2\alpha$. Densitometry of three independent experiments is illustrated in Figure 5-14(B).

Luteolin is a plant flavonoid known to inhibit tyrosine kinase activity and like other flavonoids may undergo metabolic transformation resulting in modified bioactivity (296,297). Sendobry et al. (298) identified a compound, PD146176, which lacked non-specific antioxidant activity but was reported to specifically inhibit 15-LOX while exhibiting a moderate inhibitory effect on 5- or 12-LOX activity. Therefore, total RNA from HFL-1 cells treated with PD146176 in the absence or presence of IL-1β for 8 h was analyzed by northern blot for $cPLA_2\alpha$ expression. The results in Figure 5-15(A) illustrate that PD146176 significantly decreased the IL-1β induction of $cPLA_2\alpha$ gene expression in a dose dependent manner. These results are confirmed by densitometry of the IL-1β induction from three independent experiments depicted in Figure 5-15(B). Thus far, the results demonstrate that inhibition of the LOX pathway, specifically 15-LOX and not the COX pathway is involved in the IL-1β induction of $cPLA_2\alpha$ gene expression,

supporting our hypothesis of a feed forward mechanism involved in the regulation of cPLA$_2$α gene expression. In the final section, the specific functional importance of 15-LOX activity will be verified by siRNA analysis.

Short Interfering RNA against 15-LOX Blocks the IL-1β Induction of Cytosolic PLA$_2$α Gene Expression

The previous experiments utilized putative 15-LOX inhibitors, to determine whether 15-LOX activity is required for the IL-1β-dependent induction of cPLA$_2$α. To further illustrate the importance of 15-LOX in mediating the IL-1β induction of cPLA$_2$α gene expression, knockdown of 15-LOX expression by siRNA analysis was then evaluated. Since there are two 15-LOX isoforms, their expression in HFL-1 cells following IL-1β stimulation was analyzed by real-time RT-PCR. The results indicated no basal or inducible 15-LOX1 expression in HFL-1 cells. On the other hand, HFL-1 cells exhibited basal 15-LOX2 expression and increased levels following IL-1β treatment (data not shown). Therefore an siRNA against 15-LOX2 was transfected into HFL-1 cells as indicated in the Materials and Methods and cPLA$_2$α mRNA expression was measured following 4 h of IL-1β treatment. The data in Figure 5-16 illustrates that knockdown of 15-LOX2 expression caused about a 50% decrease in cPLA$_2$α gene expression, validating the involvement of the lipoxygenase pathway, particularly 15-LOX2, in a feed forward mechanism regulating the IL-1β induction of cPLA$_2$α expression.

Discussion

The MAPK pathway and intracellular Ca^{2+} levels are known to be involved in regulating the enzymatic activity of cPLA$_2$α (104,261). This study focused on determining which aspects of the p38MAPK signaling cascade lead to the IL-1β induction of cPLA$_2$α enzymatic activation and transcriptional activation. The data confirms work presented in previous studies (104,272), illustrating that IL-1β caused a rapid activation of cPLA$_2$α phosphorylation within 10 minutes,

and that this phosphorylation event is attenuated by treatment with the p38MAPK inhibitor, SB203580. Analysis of cPLA$_2$α gene expression as a consequence of IL-1β induction was evaluated using pharmacological inhibitors of p38MAPK and mouse embryonic fibroblasts deficient for the two major p38MAPK isoforms, p38α and p38β. The results presented in Figure 5-2 to Figure 5-5, further illustrated the specific involvement of p38MAPK in the induction of cPLA$_2$α mRNA expression.

Kinases known to be involved in the p38MAPK pathway are MKK3 and MKK6 kinases, which are upstream of p38MAPK and directly involved in its phosphorylation (283), and MSK-1, a downstream target of p38MAPK (287). The results shown in Figure 5-6 to Figure 5-7 illustrate that IL-1β induces an upstream p38MAPK activator, MKK3/MKK6, which is reportedly activated by MyD88-IRAK/TRAF6 (280). A downstream target of p38MAPK, MSK-1, was also shown to be activated by IL-1β further implying involvement of p38MAPK in the cytokine-mediated induction of cPLA$_2$α (Figure 5-8). A few studies have implied that phosphorylation of cPLA$_2$α by MSK-1 may trigger the translocation of cPLA$_2$α to the perinuclear membrane, bringing the enzyme in close proximity to its substrate (299). Furthermore, Vermeulen et al. (288) illustrated that MSK-1 phosphorylates NFκB in a stimulus dependent manner and inhibition of MSK-1 significantly attenuated NFκB phosphorylation. Future studies on the activation of cPLA$_2$α gene expression may illustrate the specific role of MSK-1 in cPLA$_2$α enzymatic activation and gene transcription. Presently, looking at the data presented thus far, an interesting observation can be made, in that there is a sequential activation of MKK3/MKK6, p38MAPK, MSK-1 leading to the eventual phosphorylation of cPLA$_2$α, all happening within 10 – 30 min of IL-1β treatment. A model of depicting these events is illustrated in Figure 5-17.

Increased cPLA$_2$α enzymatic activity could potentially lead to increased cPLA$_2$α gene expression and this was proven by utilizing inhibitors of cPLA$_2$α enzymatic activity, AACOCF$_3$ and pyrrolidine. It is known that cPLA$_2$α activity is required for liberating arachidonic acid from membrane phospholipids for further metabolism by lipoxygenases and cyclooxygenase. Increased availability of arachidonic acid leads to cell-mediated production of eicosanoids which regulate numerous physiological and pathological events. The availability of these signaling molecules, whether rapidly induced within minutes or produced over a longer time scale, can exhibit varied physiological responses within the cell.

Evaluation of the involvement of downstream arachidonic acid metabolites in regulating cPLA$_2$α expression revealed that while cyclooxygenase activity was not involved in the induction, lipoxygenase activity was required for the IL-1β-dependent induction. Furthermore, using selective pharmacological inhibitors of the lipoxygenase enzymes, the data illustrated the specific involvement of 15-LOX in regulating the IL-1β induction of cPLA$_2$α (Figure 5-9 to Figure 5-15).

Further analysis of the lipoxygenase pathway by pharmacological inhibition and targeted knockdown by siRNA, illustrated the involvement of 15-LOX2 in the transcriptional activation of cPLA$_2$α (Figure 5-16). Overall this data confirmed the ability of cPLA$_2$α to regulate its own gene expression via a feed forward mechanism and illustrated the unique quality of p38MAPK to regulate both the enzymatic activation of cPLA$_2$α and transcriptional induction of cPLA$_2$α gene expression.

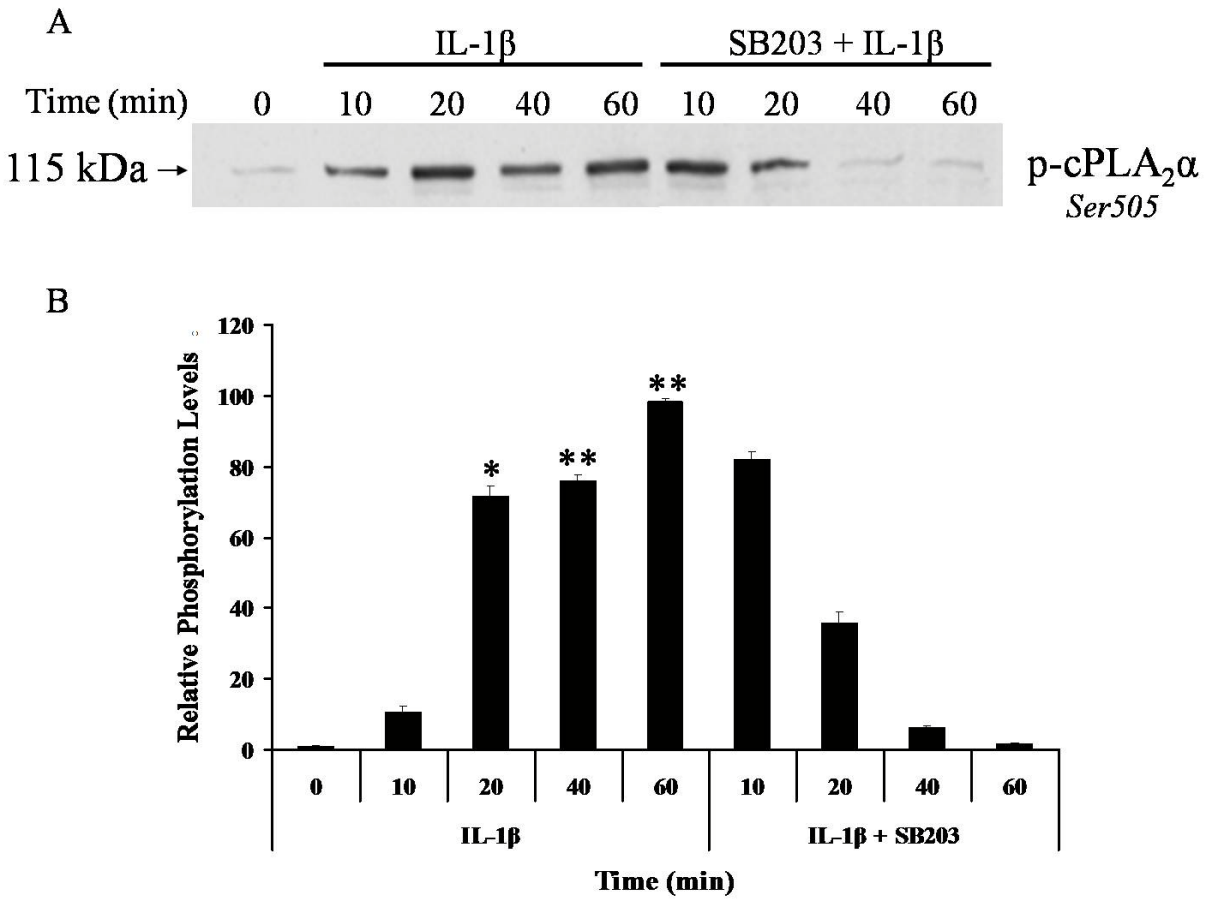

Figure 5-1. IL-1β induces cPLA$_2$α phosphorylation via the action of p38MAPK. A) HFL-1 cells were stimulated with IL-1β in the absence or presence of the p38MAPK inhibitor SB203580. Phosphorylation of cPLA$_2$α was evaluated by immunoblot analysis with a phospho-specific antibody against Ser505. B) The graph depicts densitometry of three independent experiments. The asterisk (*) indicates statistical significance with p value $\leq$ 0.05 and (**) indicates statistical significance with p value $\leq$ 0.01 as compared with the control samples.

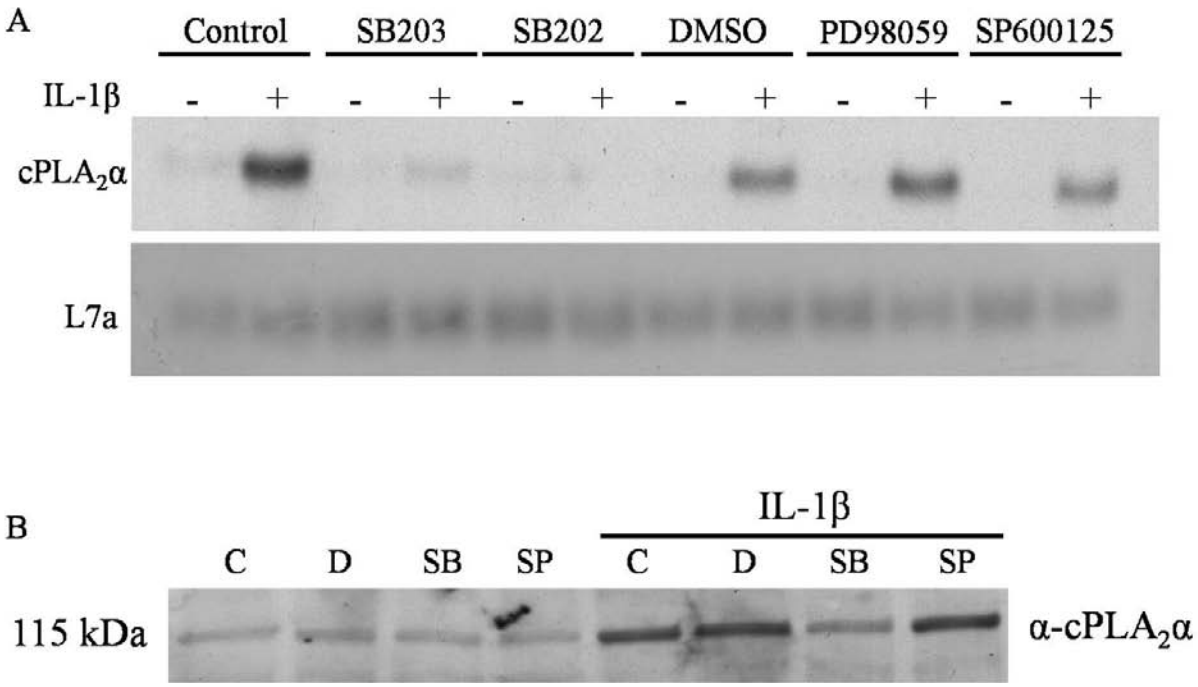

Figure 5-2. p38MAPK mediates cPLA$_2$α gene expression in an IL-1β-dependent manner. A) HFL-1 cells were exposed to p38MAPK, ERK1/2 or JNK inhibitors in the absence or presence of IL-1β for 8 h. Total RNA was isolated and analyzed by northern blot. The membrane was hybridized with radiolabeled probes for cPLA$_2$α and L7a. B) Immunoblot analysis of cPLA$_2$α protein expression in HFL-1 cells treated with the p38MAPK inhibitor, SB203580 or the JNK inhibitor, SP600125.

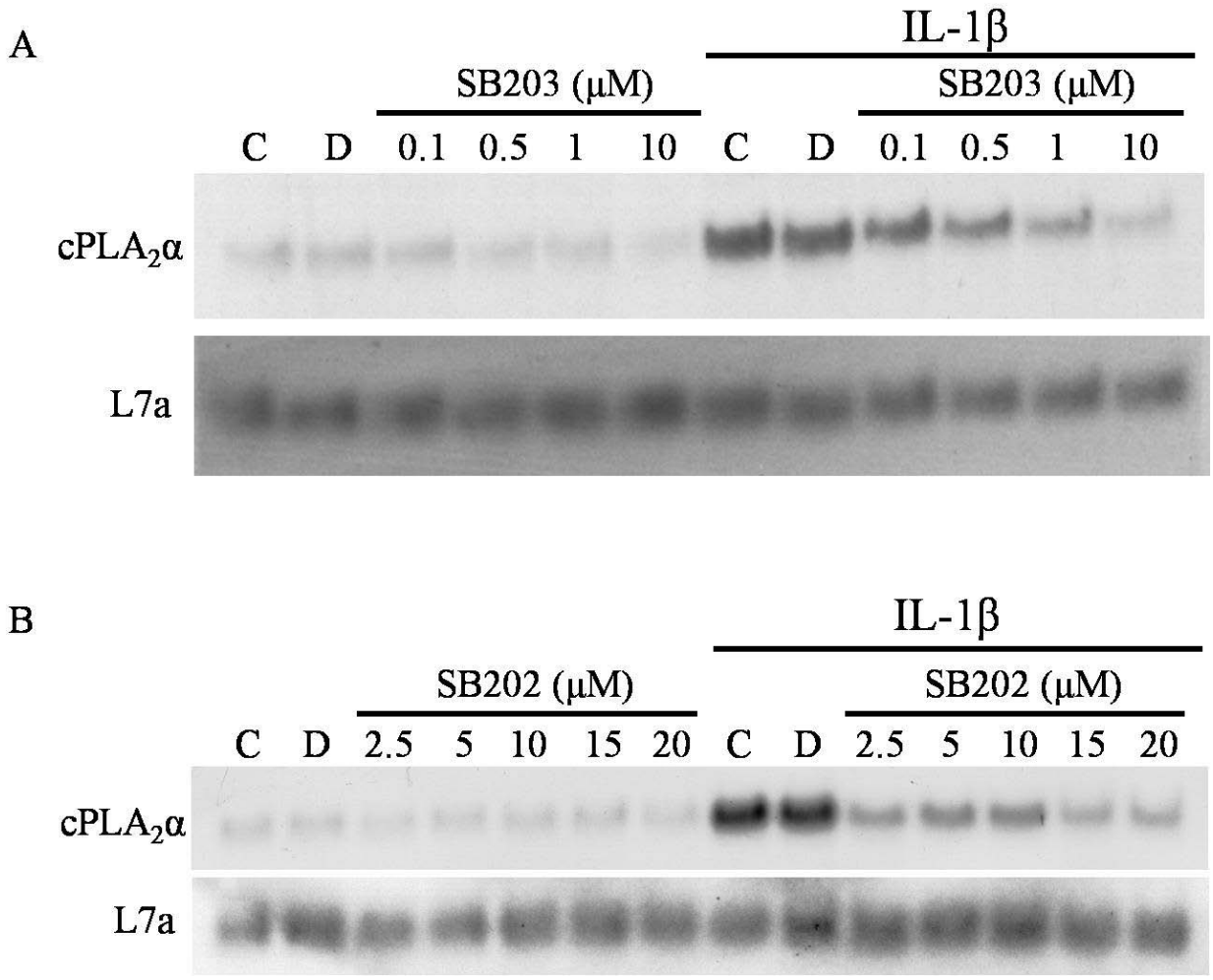

Figure 5-3. Inhibition of cPLA$_2$α enzymatic activity blocks the IL-1β induction of cPLA$_2$α gene expression: A feed forward mechanism. A) HFL-1 cells were exposed to increasing concentrations of SB203580 in the absence or presence of IL-1β for 8 h. Total RNA was extracted and subjected to northern blot analysis. The membrane was hybridized with radiolabeled probes for cPLA$_2$α and L7a (L7a serves as the loading control). B) Northern blot analysis of HFL-1 cells treated with an analog of SB203580, SB202190, in the absence or presence of IL-1β. The membrane was hybridized with radiolabeled probes for cPLA$_2$α and L7a (L7a serves as the loading control).

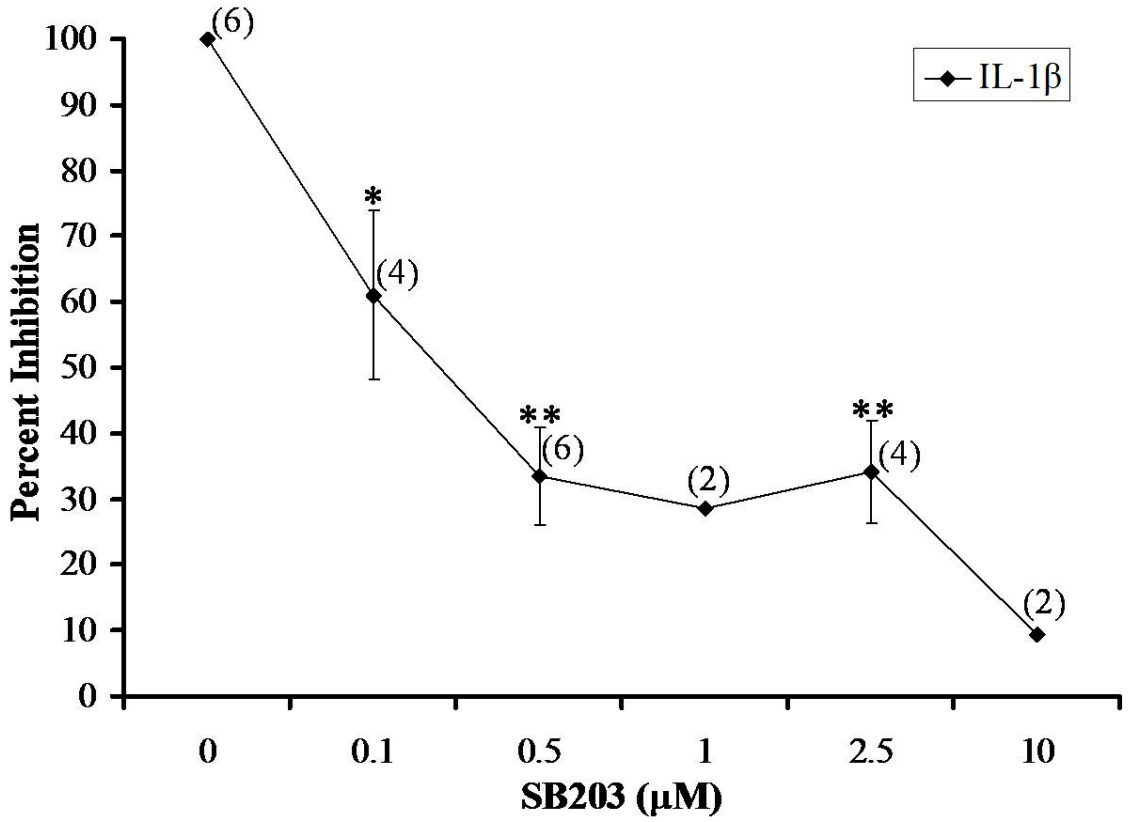

Figure 5-4. Inhibition of cPLA$_2$α enzymatic activity blocks the IL-1β induction of cPLA$_2$α gene expression: A feed forward mechanism. The graph illustrates densitometry data for HFL-1 cells treated with SB20358 in the presence of IL-1β. The number in parentheses above each point indicates the number of independent data points. The asterisk (*) indicates statistical significance with p value $\leq$ 0.05 and (**) indicates statistical significance with p value $\leq$ 0.01 as compared with the control samples.

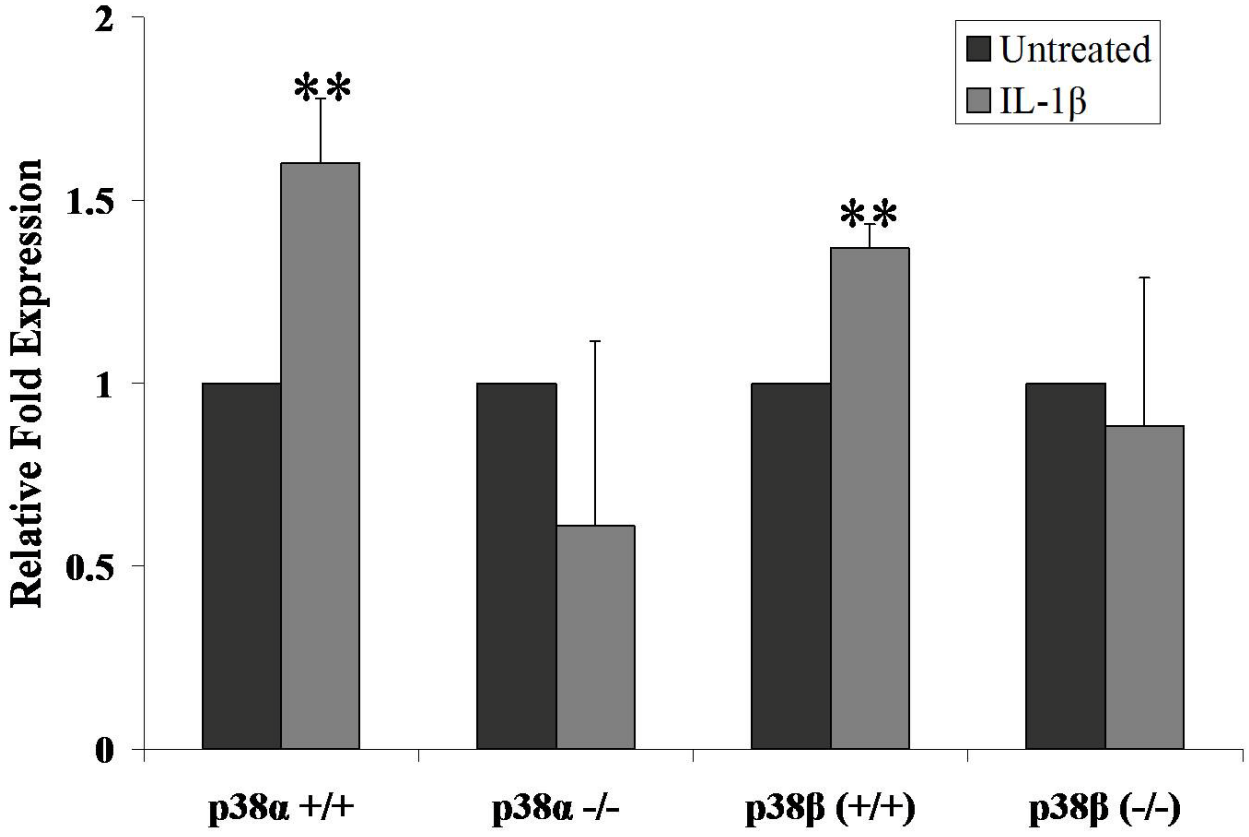

Figure 5-5. p38MAPK mediates cPLA$_2$α gene expression in an IL-1β-dependent manner. Wild-type, p38α -/- and p38β -/- MEF cells were stimulated with IL-1β for 8 h. Total RNA was isolated and subjected to real-time RT-PCR analysis to detect cPLA$_2$α gene expression. The cPLA$_2$α/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of four independent experiments, where the data points are represented as mean ± SEM. The asterisk (**) indicates statistical significance with p value $\leq$ 0.01 as compared with the control sample.

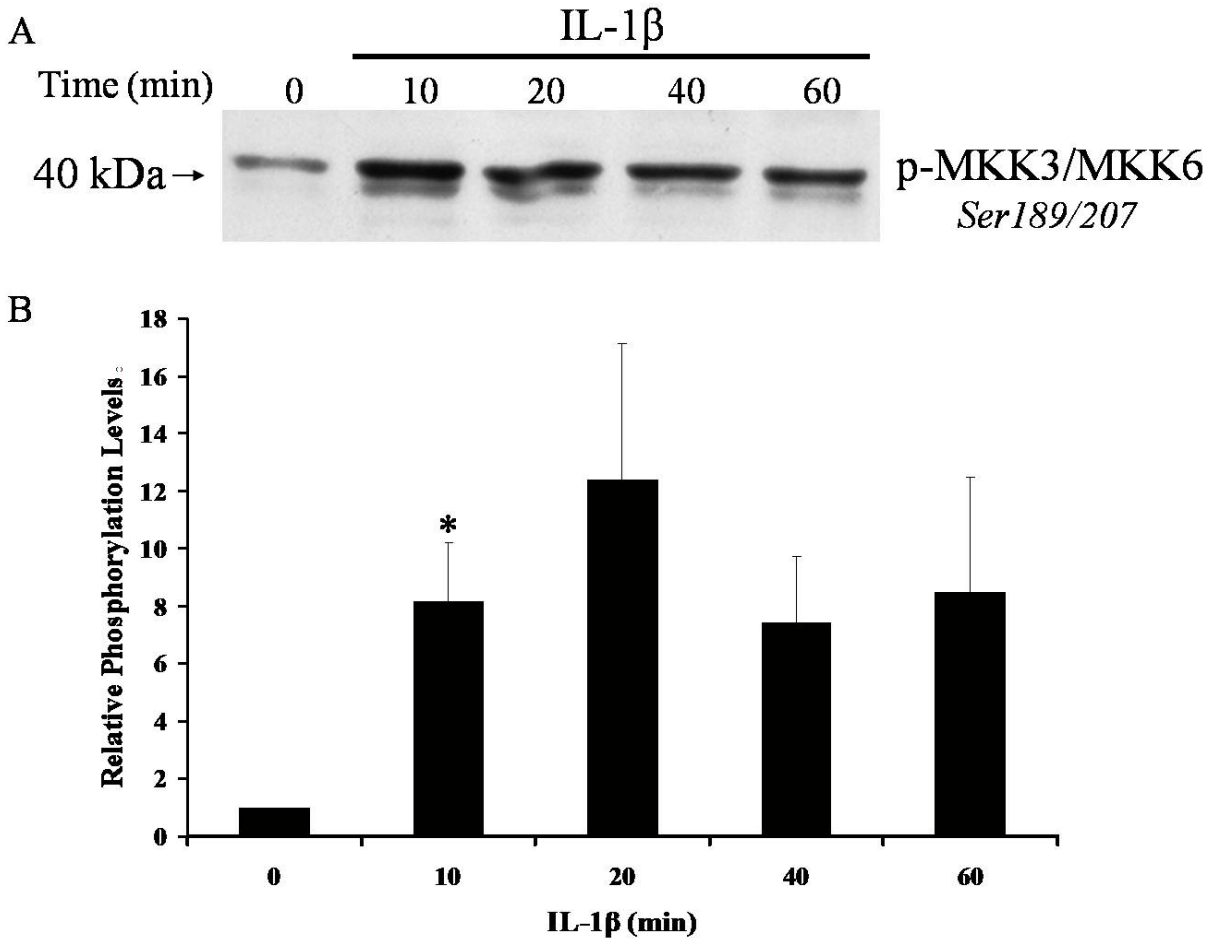

Figure 5-6. Phosphorylation of MKK3/MKK6 is induced by IL-1β. A) HFL-1 cells were stimulated with IL-1β and phosphorylation of MKK3/MKK6 was evaluated by immunoblot analysis with a dual phospho-specific antibody recognizing Ser189 (MKK3) and Ser207 (MKK6). B) The graph depicts densitometry of three independent experiments. The asterisk (*) indicates statistical significance with p value $\leq$ 0.05 as compared with the control samples.

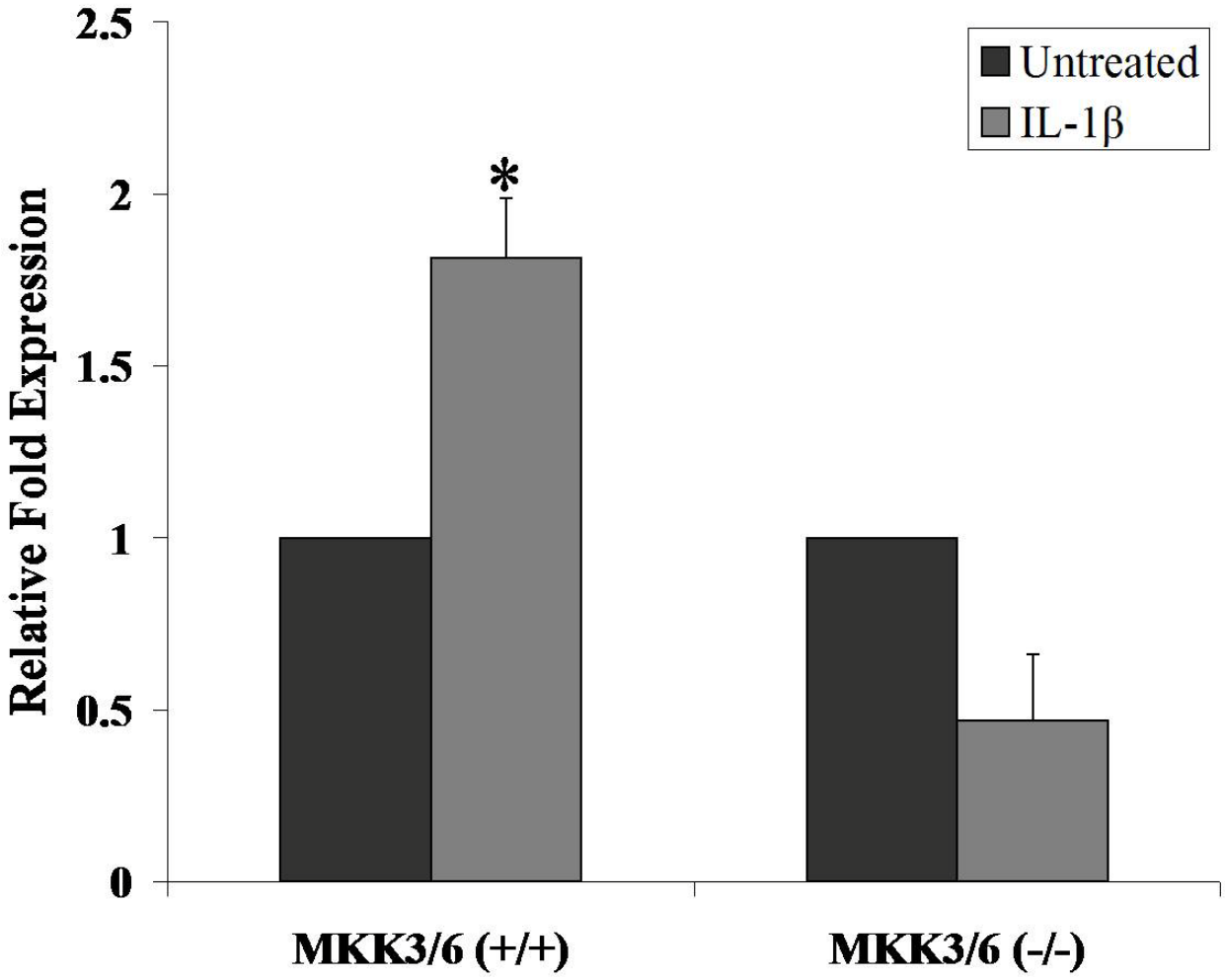

Figure 5-7. MKK3/MKK6 mediates $cPLA_2\alpha$ gene expression in an IL-1β-dependent manner. Wild-type and MKK3/6 -/- MEF cells were treated with IL-1β for 8 h. Total RNA was isolated and subjected to real-time RT-PCR analysis to detect $cPLA_2\alpha$ expression. The $cPLA_2\alpha$/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of three independent experiments, where the data points are represented as mean ± SEM. The asterisk (*) indicates statistical significance with p value $\leq$ 0.05 as compared with the control sample.

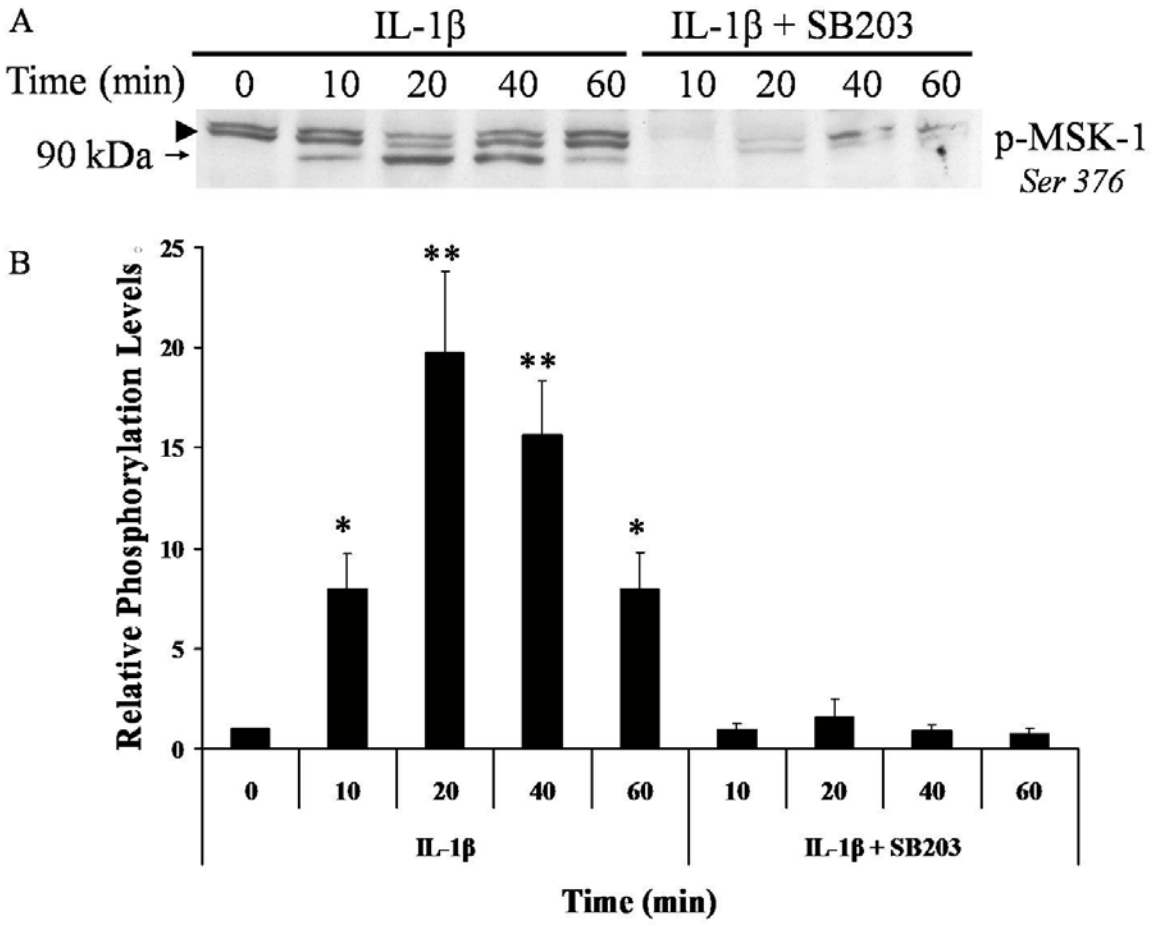

Figure 5-8. Phosphorylation of MSK-1 is induced by IL-1β. A) HFL-1 cells were stimulated with IL-1β in the absence or presence of the p38MAPK inhibitor, SB203580. Phosphorylation of MSK-1 was evaluated by immunoblot analysis with a phospho-specific antibody against Ser376. The arrow head (►) indicates an unspecific interaction. B) The graph depicts densitometry of three independent experiments. The asterisk (*) indicates statistical significance with p value $\leq$ 0.05 and (**) indicates statistical significance with p value $\leq$ 0.01 as compared with the control samples.

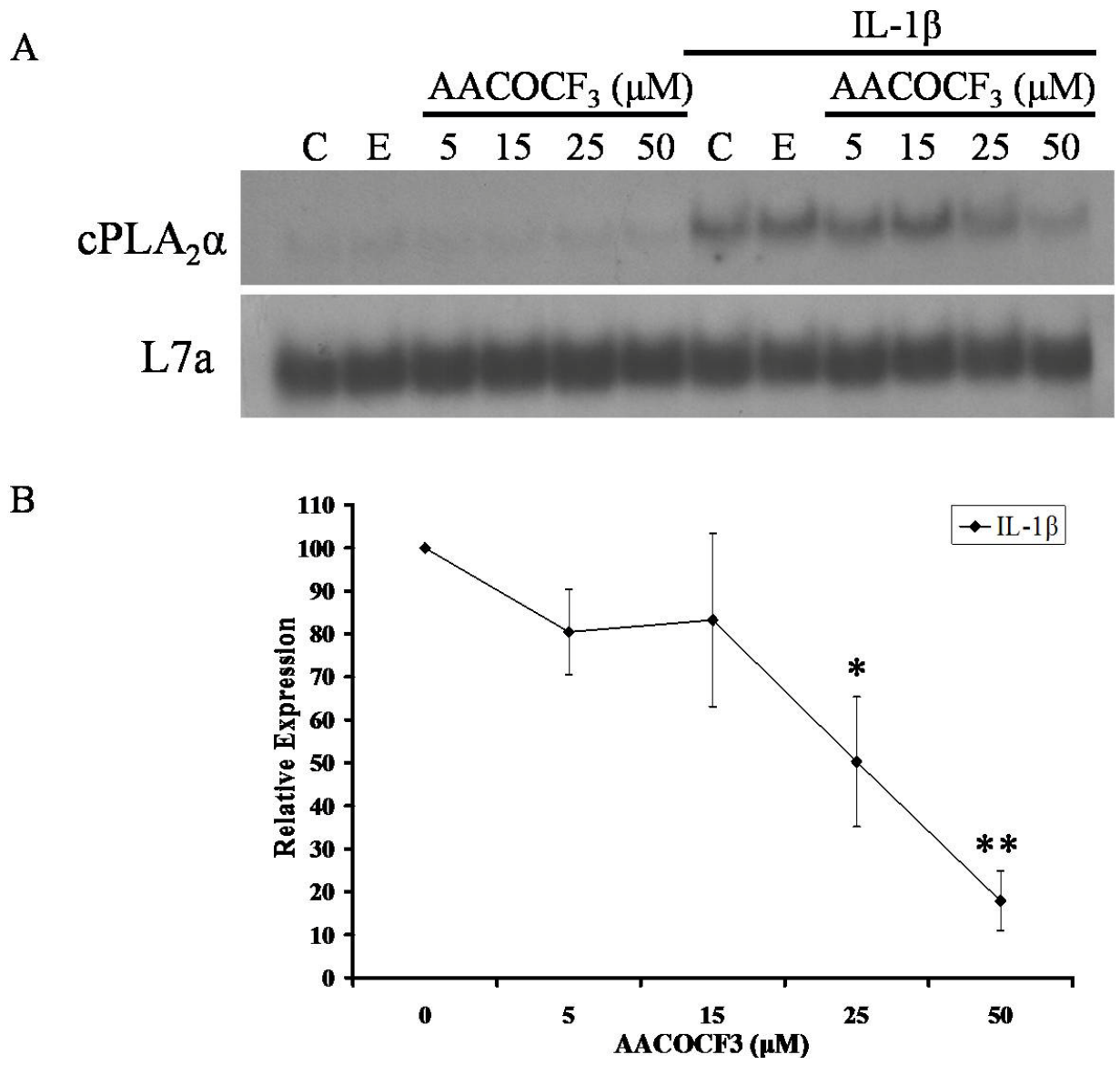

Figure 5-9. The lipoxygenase pathway but not cyclooxygenase pathway is necessary for $cPLA_2\alpha$ expression: Inhibition of $cPLA_2\alpha$ enzymatic activity. A) HFL-1 cells were exposed to an inhibitor of $cPLA_2\alpha$ enzymatic activity, $AACOCF_3$ in the absence or presence of IL-1β for 8 h. Total RNA was isolated and analyzed by northern blot. The membrane was hybridized with radiolabeled probes for $cPLA_2\alpha$ and L7a. B) The graph depicts a summary of three independent experiments, where the data points are represented as mean ± SEM. The asterisk (*) indicates statistical significance with p value $\leq$ 0.05 and (**) indicates statistical significance with p value $\leq$ 0.01 as compared with the control sample.

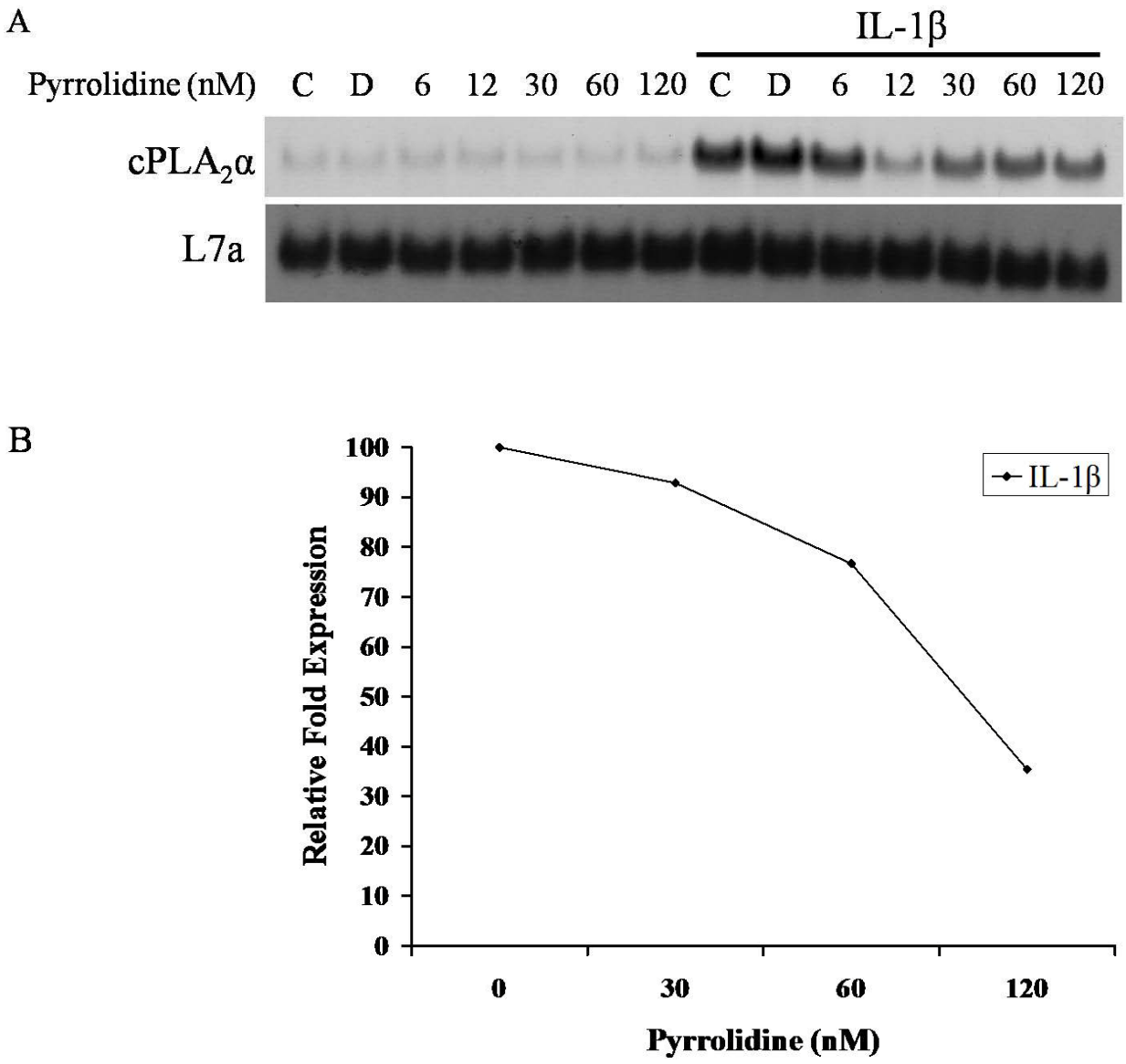

Figure 5-10. The lipoxygenase pathway but not cyclooxygenase pathway is necessary for $cPLA_2\alpha$ expression: Inhibition of $cPLA_2\alpha$ enzymatic activity. A) Northern analysis of HFL-1 cells which were treated with pyrrolidine, an inhibitor of $cPLA_2\alpha$ enzymatic activity, in the absence or presence of IL-1β for 8 h. The membrane was hybridized with radiolabeled probes for $cPLA_2\alpha$ and L7a. B) Densitometric analysis of two independent experiments where the data points are represented as an average.

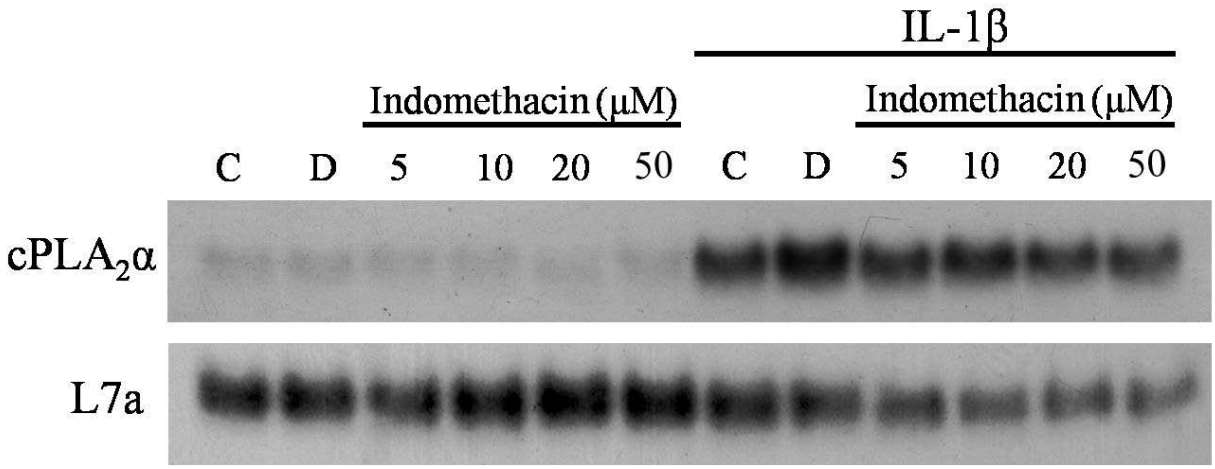

Figure 5-11. The lipoxygenase pathway but not cyclooxygenase pathway is necessary for $cPLA_2\alpha$ expression: Inhibition of COX. A) HFL-1 cells were treated with the non-selective COX inhibitor, indomethacin, in the absence or presence of IL-1β for 8 h. Total RNA was analyzed by northern blot to detect $cPLA_2\alpha$ expression. The membrane was hybridized with radiolabeled probes for $cPLA_2\alpha$ and L7a.

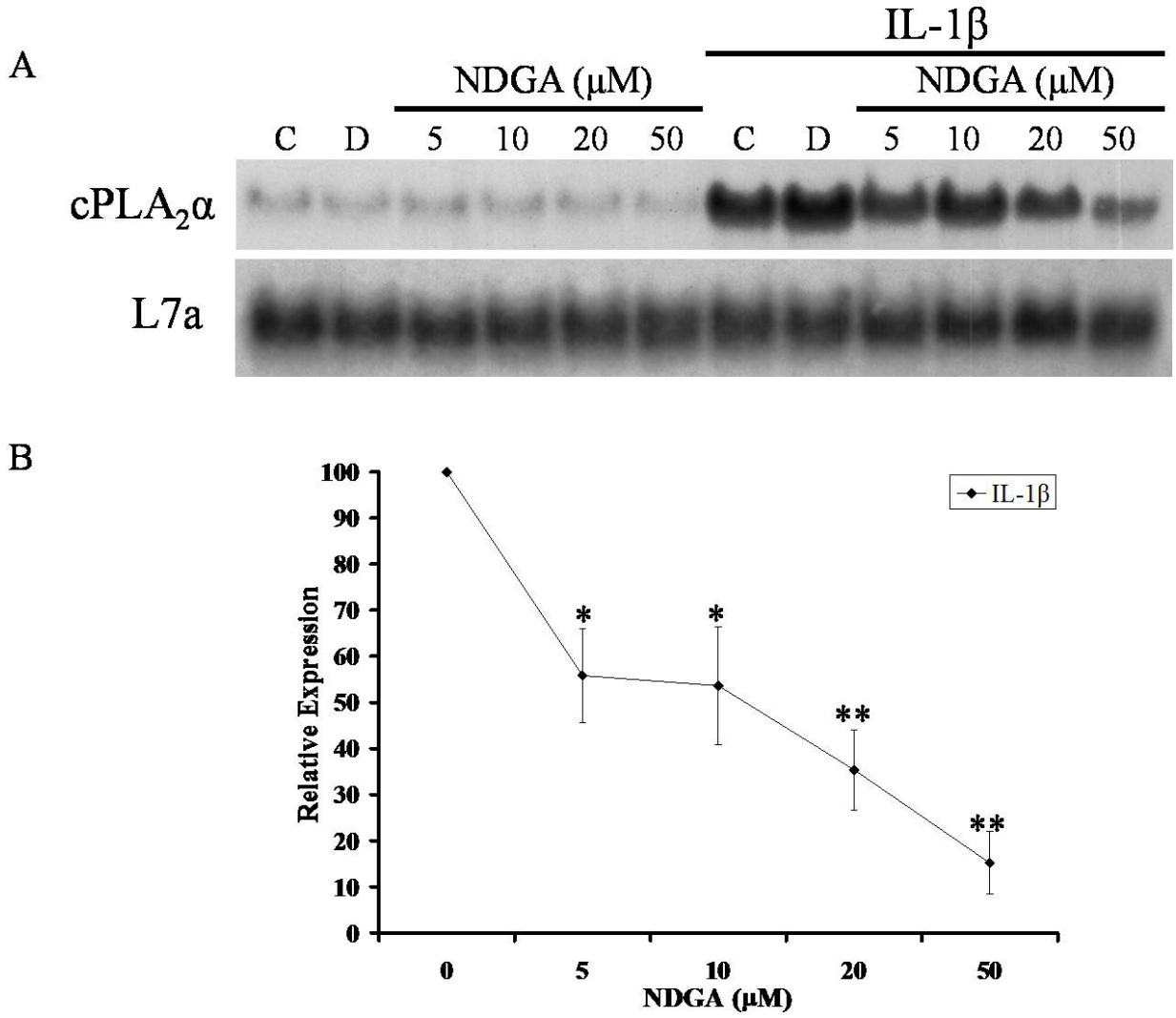

Figure 5-12. The lipoxygenase pathway but not cyclooxygenase pathway is necessary for $cPLA_2\alpha$ expression: Inhibition of LOX. A) Northern analysis of HFL-1 cells treated with the selective LOX inhibitor, NDGA, in the absence or presence of IL-1β for 8 h. The membrane was hybridized with radiolabeled probes for $cPLA_2\alpha$ and L7a. B) Densitometry of three independent experiments, where the data points are represented as mean ± SEM. The asterisk (*) indicates statistical significance with p value $\leq$ 0.05 and (**) indicates statistical significance with p value $\leq$ 0.01 as compared with the control sample.

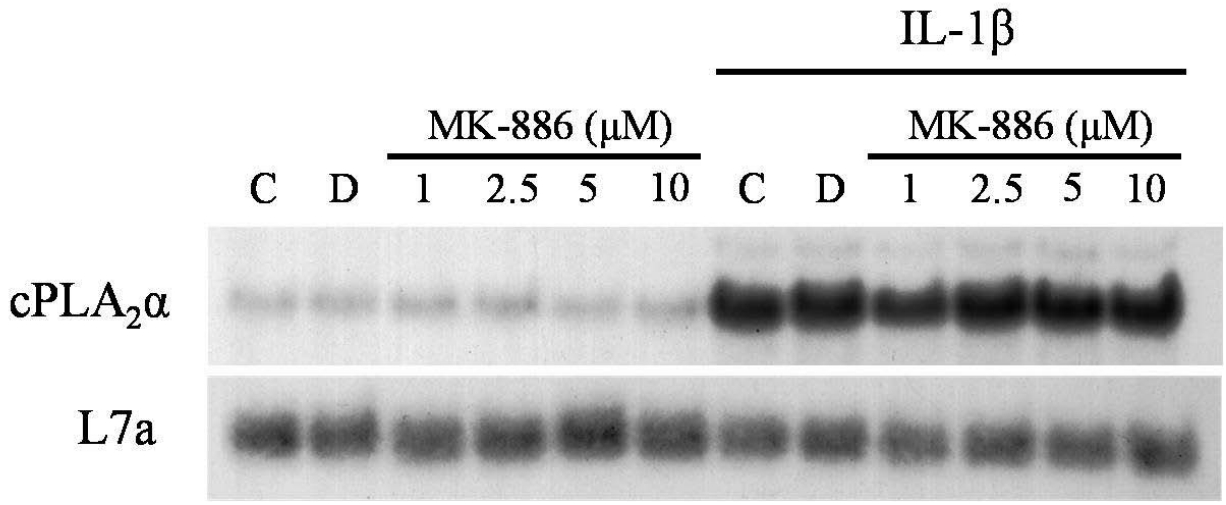

Figure 5-13. The lipoxygenase pathway but not cyclooxygenase pathway is necessary for $cPLA_2\alpha$ expression: Inhibition of 5-LOX. HFL-1 cells were treated with a 5-LOX inhibitor, MK-886, in the absence or presence of IL-1β for 8 h. Total RNA was analyzed by northern blot to detect $cPLA_2\alpha$ expression. The membrane was hybridized with radiolabeled probes for $cPLA_2\alpha$ and L7a.

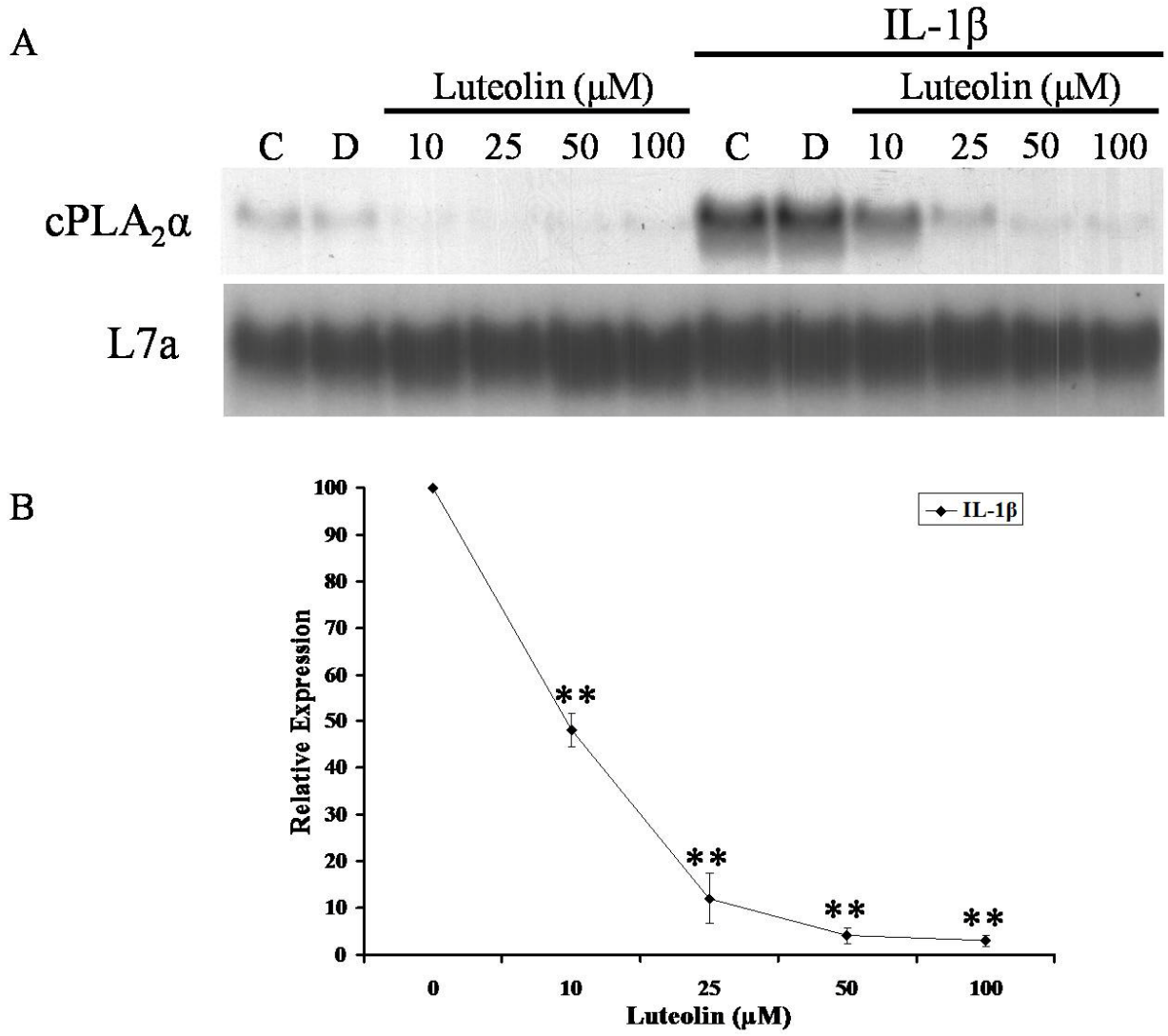

Figure 5-14. The lipoxygenase pathway but not cyclooxygenase pathway is necessary for cPLA$_2$α expression: Inhibition of 12/15-LOX. A) Northern analysis of HFL-1 cells treated with a general LOX inhibitor, luteolin, in the absence or presence of IL-1β for 8 h. The membrane was hybridized with radiolabeled probes for cPLA$_2$α and L7a. B) Densitometry of three independent experiments, where the data points are represented as mean ± SEM. The asterisk (*) indicates statistical significance with p value $\leq$ 0.05 and (**) indicates statistical significance with p value $\leq$ 0.01 as compared with the control sample.

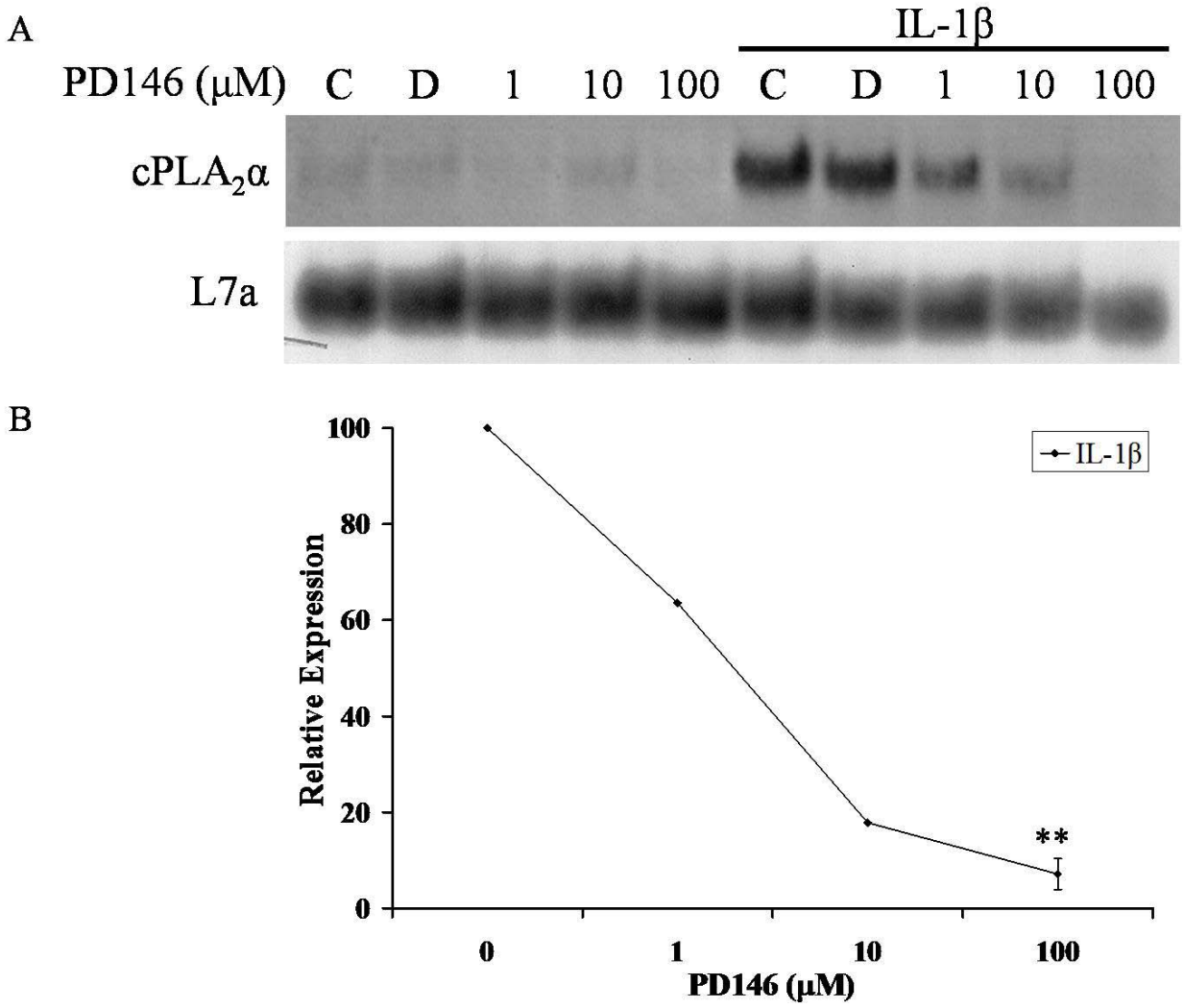

Figure 5-15. Pharmacological inhibition of 15-LOX and siRNA against 15-LOX activity blocks the IL-1β induction of $cPLA_2\alpha$ gene expression. A) HFL-1 cells were treated with the 15-LOX specific inhibitor, PD146176, in the absence or presence of IL-1β for 8 h. Total RNA was analyzed by northern blot to detect $cPLA_2\alpha$ expression. The membrane was hybridized with radiolabeled probes for $cPLA_2\alpha$ and L7a. B) Densitometry of three independent experiments, where the data points are represented as mean ± SEM. The asterisk (**) indicates statistical significance with p value ≤ 0.01 as compared with the control sample.

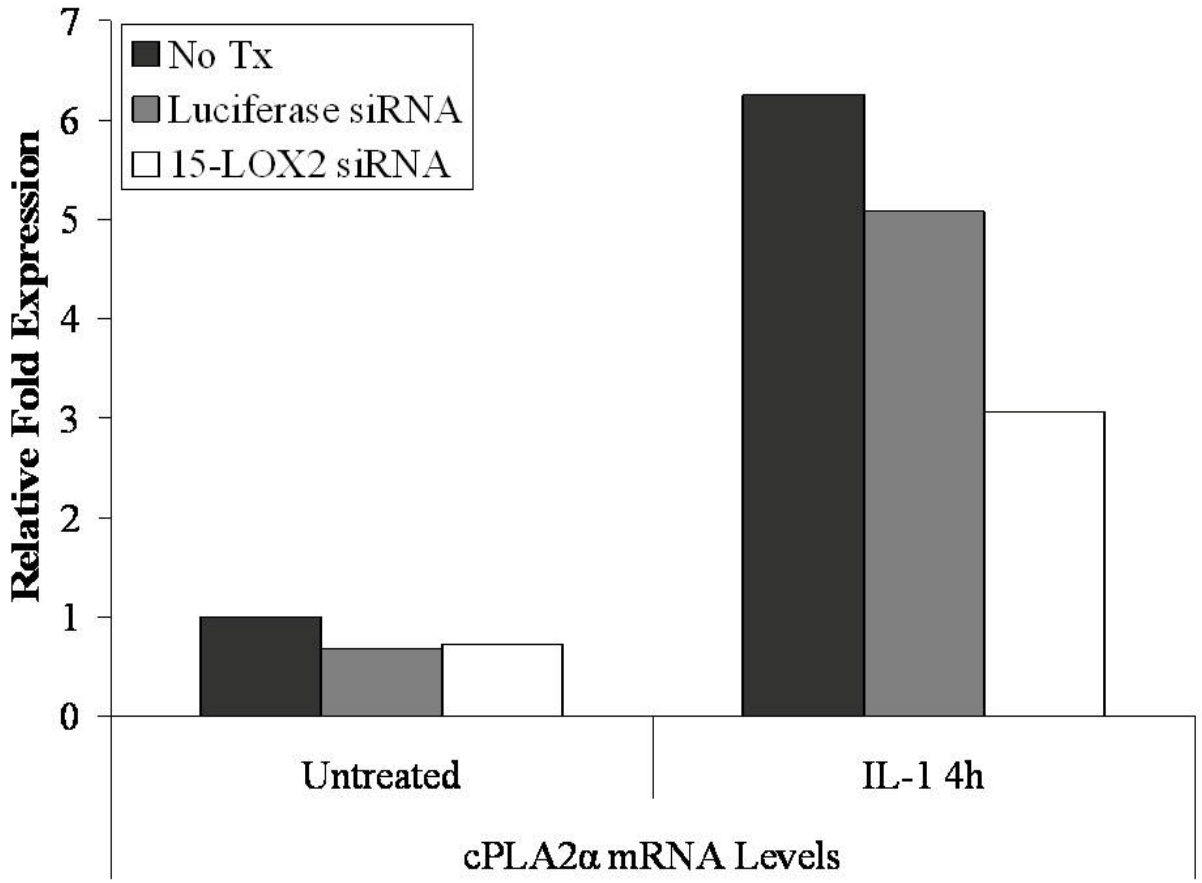

Figure 5-16. Pharmacological inhibition of 15-LOX and siRNA against 15-LOX activity blocks the IL-1β induction of $cPLA_2\alpha$ gene expression. HFL-1 cells were transfected with a control siRNA targeting Luciferase, or with an siRNA specifically targeting human 15-LOX2, with or without 4h IL-1β treatment. Total RNA was extracted and subjected to real-time RT-PCR analysis to detect either $cPLA_2\alpha$ or cyclophilin A mRNA. The $cPLA_2\alpha$/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of two independent experiments.

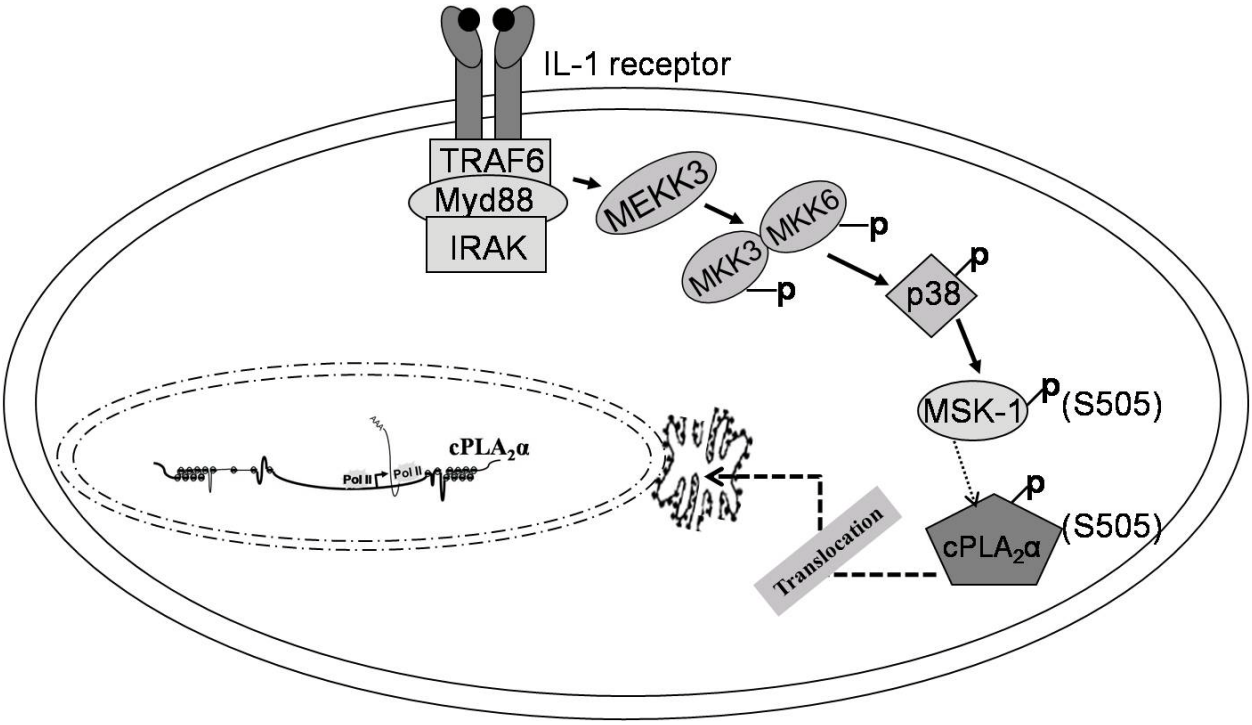

Figure 5-17. Model of $cPLA_2\alpha$ activation. IL-1β binds to its receptor activating MyD88/IRAK/TRAF6 and triggering the phosphorylation of MEKK3. The dual kinase MKK3/MKK6 is activated by phosphorylation and in turn phosphorylates, p38MAPK. p38MAPK goes on to phosphorylate MSK-1 which ultimately leads to the phosphorylation and enzymatic activation of $cPLA_2\alpha$. The activated enzyme translocates to the peri-nuclear membrane and is able to mediate downstream transcriptional events.

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

PGE_2 has been well characterized and is known to play a role in a number of biological and pathophysiological functions. As a downstream product of arachidonic acid metabolism, it was initially thought to be a direct by-product of COX metabolism of the central prostanoid intermediate, PGH_2. In the late 90's Jakobsson et al. (192) identified a terminal prostaglandin E synthase and showed that this enzyme was directly responsible for the production of PGE_2 and was induced by the pro-inflammatory cytokine, IL-1β. A decade later, numerous studies illustrated the importance of mPGES-1 in PGE_2 production and demonstrated that a stress response factor, Egr-1, is capable of binding to the proximal promoter region of mPGES-1 thus driving its inducible expression (207,234,237,300). Since that time, no other studies have clarified the exact mechanisms involved in the cytokine-dependent regulation of mPGES-1 gene expression and aside from the proximal promoter no other regulatory elements have been identified. Therefore, the goal of the current study was to examine the underlying mechanisms surrounding the induction of mPGES-1 gene expression by pro-inflammatory cytokines.

In my initial studies, I examined the induction of mPGES-1 gene transcription as a consequence of IL-1β stimulation and the results illustrated that both the mRNA and protein levels were strongly up-regulated in the presence of IL-1β (Figure 3-1 to Figure 3-4). Degousee et al. (233) illustrated that the induced mPGES-1 message had a half-life of ~6 h compared to ~3 h in the un-induced state in cardiomyocytes. In a parallel study, I analyzed decay of the induced message following stimulus removal; the results showed that the message had a half-life of about ~6 h in human lung fibroblasts (Figure 3-5). Further, I was able to demonstrate the requirement of *de novo* transcription for the cytokine-mediated induction of mPGES-1 gene expression, by

actinomycin D treatment and the measurement of hnRNA levels, a pre-spliced mRNA intermediate (Figure 3-6 and Figure 3-7).

Analysis of two mPGES-1 promoter fragments illustrated basal promoter activation in the absence of stimulus and a subsequent increase in activity following IL-1β stimulation. Functional analysis of the wild type mPGES-1 promoter fragment (1.1 kb) and a mutant construct harboring an Egr-1 deletion, confirmed the involvement of Egr-1 in regulating promoter activation corroborating the studies previously conducted by other groups (Figure 3-8 to Figure 3-9) (206,207). Alternatively, I used computer analysis to predict the location of transcription factor binding sites within the promoter and identified a potential binding site for C/EBPβ. Deletion of this site revealed that it did not contribute to the basal or induced promoter activation.

In the absence of other data or regulatory studies in the literature, we hypothesized that there must be additional regulatory elements within or near the mPGES-1 gene that are involved in regulating its expression and thus achieving the level of induction observed by measuring steady-state increases. The first approach was to generate fragments of the entire mPGES-1 locus, subclone each fragment into the hGH construct and evaluate the reporter activity, a strategy based on our lab's experience in identifying internal cytokine-dependent regulatory elements. Therefore, I examined fragments internal to the gene and found no regulatory elements that significantly contributed to overall promoter activation by IL-1β (Figure 3-10). Further, the overall induction with each of these fragments was similar to that of the promoter alone, approximately ~1.5 – 2 fold.

Taking another approach, DNase I hypersensitive site analysis was utilized to identify potential regulatory regions (Figure 3-11 and Figure 3-12). This method allows for the rapid

detection of structural alterations or open chromatin regions associated with hypersensitive sites harboring regulatory factors and their analogous binding sites. These open regions can then be further evaluated for functional significance as it relates to the IL-1β induction.

The analysis revealed the existence of two potential regulatory regions or constitutive hypersensitive sites, one mapping to ~-0.3 kb and the other mapping at ~ -8.6 kb. Unlike inducible hypersensitive sites, constitutive hypersensitive sites are known to be associated with the promoter region of genes and subsequent transcriptional activation. At times, finer analysis of these regions reveal the existence of regulatory regions that are potentially involved in regulating gene expression (301). The first site mapped to the region of Egr-1 binding which was previously analyzed by site-directed mutagenesis of the mPGES-1 promoter construct (Figure 3-8 and Figure 3-9). Functional analysis of the second HS site illustrated that the fragment exhibits enhancer-like characteristics, functioning in an orientation independent manner and activating transcription through a heterologous promoter in an IL-1β-dependent manner. Also this site contained both a basal and inducible element involved in the regulation of mPGES-1 expression (Figure 4-1 to Figure 4-6). Furthermore, combining the level of induction seen in the induced lane of the promoter+enhancer construct (~9 fold) with the un-induced lane of the promoter alone construct, recapitulates the level of induction of the endogenous gene by northern blot and real-time RT-PCR.

Contrary to published reports which indicated that Egr-1 is the sole factor regulating the induction of mPGES-1 gene expression; deletion analysis of the inducible enhancer element together with site-directed mutagenesis, siRNA and the availability of wild type and knockout MEF cells revealed the involvement of another transcription factor, C/EBPβ as a key mediator of the IL-1β-dependent induction of mPGES-1 gene expression, the existence of which has not been

previously reported (Figure 4-7 to Figure 4-13). Further analysis of both Egr-1 and C/EBPβ by chromatin immunoprecipitation indicated that Egr-1 bound to the promoter in an IL-1β-independent manner while C/EBPβ bound in a cytokine-dependent manner to the enhancer element (Figure 4-14 to Figure 4-15). This analysis also illustrated that RNA Polymerase II bound to the mPGES-1 promoter in an IL-1β manner.

It is not known whether Egr-1 and C/EBPβ are capable of interacting and therefore a co-immunoprecipitation analysis was conducted and revealed that these factors are capable of interacting independent of cytokine treatment (Figure 4-16). In lieu of other known data, our model of IL-1β activation suggests that under basal conditions, Egr-1 is bound to the promoter, while C/EBPβ is bound at the enhancer. Also, in the absence of IL-1β, RNA Polymerase II is bound to the promoter. Following IL-1β treatment, there is a significant increase in RNA Polymerase II binding at the promoter and C/EBPβ binding at the enhancer. At some point there is cross talk between Egr-1 and C/EBPβ leading to the up-regulation of mPGES-1 expression.

Overall, this study focused on delineating the mechanisms involved in regulating the physiological levels of mPGES-1. It also illustrated the need for a concise examination of the entire mPGES-1 locus. This work revealed the involvement of another transcription factor, C/EBPβ aside from Egr-1, in mediating the IL-1β-dependent induction of mPGES-1. Most relevantly, the data presented thus far illustrate that regions outside of the proximal promoter are required to achieve the full expression seen by IL-1β. Hopefully this work will aid in the development of anti-inflammatory drugs aimed at inhibiting mPGES-1 enzymatic activation.

$cPLA_2\alpha$ is the enzyme responsible for liberating arachidonic acid from membrane phospholipids for downstream metabolism. Enzymatic activation of $cPLA_2\alpha$ is regulated by intracellular calcium levels and MAP kinase activity (84,91,104,109). Initially a few groups

illustrated that cPLA$_2$α enzymatic activity is rapidly induced within 30 min to 1 h, following treatment with pro-inflammatory cytokines (272). Further, p38MAPK activation is known to play a role in the enzymatic activation of cPLA$_2$α (109,275,302). As such, our studies focused on delineating the involvement of kinase pathways and we hypothesized that downstream arachidonic acid metabolites may be involved in regulating cPLA$_2$α gene expression. Since many studies indicated that kinase activity is involved in mediating cPLA$_2$α enzymatic activity, our initial studies utilized specific kinase inhibitors to determine some of the pathways involved. Our data revealed that IL-1β caused rapid phosphorylation of cPLA$_2$α, which was subsequently blocked by inhibition of p38MAPK. Using inhibitors to ERK, JNK and MAPK, it was found that inhibition of p38MAPK attenuated the IL-1β-induced activation of cPLA$_2$α gene transcription (Figure 5-1 to Figure 5-4). Also, analysis of p38α and p38β MEFs further illustrated the importance of p38MAPK in cPLA$_2$α activation (Figure 5-5). Interestingly, MKK3/MKK6, a kinase known to phosphorylate p38MAPK (283), showed increased levels of phosphorylation following IL-1β treatment (Figure 5-6). This was further supported by data from MKK3/MKK6 MEF cells which illustrated that in the absence of the dual kinases, MKK3/MKK6, the IL-1β induction of cPLA$_2$α gene transcription was attenuated (Figure 5-7).

Another kinase, MSK-1 which is downstream of p38MAPK and identified as a target of p38MAPK activity (285), was analyzed by immunoblot and was phosphorylated following IL-1β treatment (Figure 5-8). Together the data suggests that IL-1β induces activation of MKK3/MKK6 which in turn phosphorylates p38MAPK leading to the activation of MSK-1 and eventually cPLA$_2$α enzymatic activation, all happening within 10 – 30 min post treatment (Figure 5-17). Although our data could not illustrate the direct involvement of MSK-1 in the

activation of cPLA$_2$α, Aimond et al. (303) illustrated this event in cardiomyctes, analyzing cPLA$_2$α protein expression following treatment with an inhibitor of MSK-1, Ro318220.

We then hypothesized that products of arachidonic acid metabolism may play a role in the IL-1β induction of cPLA$_2$α. First, inhibition of cPLA$_2$α enzymatic activity by AACOCF$_3$ and pyrrolidine illustrated that cPLA$_2$α enzymatic activity was required for transcriptional activation by IL-1β (Figure 5-9 and Figure 5-10). Analysis of downstream arachidonic acid metabolites revealed that COX activity was not involved in the IL-1β induction of cPLA$_2$α gene expression illustrated by inhibition with indomethacin (Figure 5-11). Using inhibitors to the lipoxygenase pathway illustrated the specific involvement of 15-LOX in the activation of cPLA$_2$α expression (Figure 5-12 to Figure 5-15). Real-time RT-PCR analysis of 15-LOX1 and 15-LOX2 expression in HFL-1 cells showed that only 15-LOX2 is expressed in these cells (data not shown). Therefore, utilizing an siRNA against 15-LOX2 confirmed the involvement of this lipoxygenase in the IL-1β activation of cPLA$_2$α gene expression (Figure 5-16). Overall the data illustrated the role of a feed forward mechanism involved in regulating the enzymatic activity and transcriptional induction of cPLA$_2$α, along with the direct involvement of the p38MAPK signaling pathway. Coupled with current work being conducted in our lab, we will hopefully be able to contribute further information on the enzymatic and transcriptional activation of cPLA$_2$α expression.

Future Directions

Like previous studies, my analysis of the mPGES-1 promoter region revealed increased activation following IL-1β but there was a visible difference in the overall induction. Densitometric analysis revealed that the 1.1 kb fragment elicited a 2.5 fold increase in reporter expression versus a 1.5 fold increase seen with the 0.6 kb fragment implying that there is a potential regulatory element between -1.1 kb and -434 kb. Computer analysis of this region

identified a C/EBP site which was later ruled out. Further, Egr-1 was shown to be important for induced gene expression by promoter deletion, conducted by our group and others. Another group conducted siRNA analysis of Egr-1 and found that there was a 50% reduction in induced promoter activation. The data presented in Chapter 3 confirmed the involvement of Egr-1 on the IL-1β induction of mPGES-1 and I believe that Egr-1 -/- MEF cells may reveal in the presence of IL-1β, that mPGES-1 levels are significantly increased more than ~2 fold (which is seen with promoter fragments); indicating the involvement of another regulatory factory that co-operatively interacts with Egr-1 to regulate promoter activation. Therefore, further analysis of the promoter fragment is needed to identify other potential factors involved in the activation of the mPGES-1 promoter.

Analysis of the mPGES-1 gene by DNase I led to the discovery of two constitutive hypersensitive sites. Functional analysis of the second site illustrated basal and inducible activity. The inducible activity was further characterized to a 500 bp region and the involvement of the transcription factor C/EBPβ was reported but no other work was done on the basal element. It is possible that like the inducible element, a single or multiple transcription factors are co-operatively regulating the basal expression of this fragment. Complete mapping and functional analysis of the basal element are needed. ChIP and co-IP analysis revealed that Egr-1 can interact with C/EBPβ and RNA Polymerase II binds inducibly to the promoter. It is unclear whether Egr-1 is involved in recruiting RNA Polymerase II to the promoter or even if they can interact. I believe that Egr-1 is potentially interacting with members of the pre-initiation complex and as such is involved in the recruitment of RNA Polymerase II to the promoter. To test this hypothesis, I suggest further analysis of Egr-1/RNA Polymerase II interaction by ChIP

or co-immunoprecipitation followed by immunoblot analysis with antibodies specific to known members of the pre-initiation complex.

The cPLA$_2$α study illustrated the involvement of MKK3/MKK6 and p38MAPK by immunoblot and MEF cell analyses. Although MSK-1 is reportedly involved, the data provided thus far only supports part of our model of cPLA$_2$α activation. I was unable to obtain mouse embryonic fibroblasts deficient for MSK-1 and my hypothesis is that analogous to the transcriptional induction of cPLA$_2$α gene expression, cPLA$_2$α protein expression would be activated in the wild type MSK-1 MEFs following IL-1β induction but not the MSK-1 -/- MEFs. Therefore, further analysis of cPLA$_2$α expression in MSK-1 MEF cells is needed to complete the story surrounding p38MAPK, MSK-1 phosphorylation and activation of cPLA$_2$α.

The siRNA analysis implicated 15-LOX2 in the regulation of cPLA$_2$α gene expression, while MEF cells for 15-LOX2 do not exist as yet, I believe IL-1β would stimulate cPLA$_2$α activation and transcriptional induction in the wild type MEFs but only cPLA$_2$α enzymatic activity would be induced in the 15-LOX2 -/- cells. Data not presented in this dissertation suggested the potential involvement of NFκB in regulating cPLA $_2$α gene expression and as such current studies are underway in the lab by Dr. Kimberly Aiken, to characterize the regulation of cPLA$_2$α gene expression by IL-1β induction.

APPENDIX
EVALUATION OF EFFECTS OF A DIFFERENT PRO-INFLAMMATORY CYTOKINE, TNF-ALPHA ON MICROSOMAL PROSTAGLANDIN SYNTHASE-1

Introduction

Analysis of Microsomal PGES-1 Expression and Promoter Activity in Human Breast Cancer Cells

Breast cancer is one of the leading causes of death in women and the third leading cause of cancer deaths in the US (304). At the cellular level, many factors are involved in the regulation, genetic and epigenetic changes associated with breast cancer, invasiveness and eventual prognosis of this disease. Some breast cancers can be classified based on estrogen receptor status, ER+ or ER- and the hormone estrogen is speculated to stimulate the proliferation of breast cancer cells (305,306).

Estrogen has been shown to up-regulate a number of genes that are involved in the proliferation and survival of breast cancer cells. Aromatase activity is known to induce estrogen biosynthesis in breast cancer and PGE_2, a bi-product of arachidonic acid metabolism regulates aromatase expression (307,308). Two studies revealed mPGES-1 is expressed in breast cancer cell lines versus normal tissue and further, mPGES-1 expression in tumors is associated with estrogen up-regulation (223,309).

Recently, Frasor et al. (209) delineated that the inducible PGE_2 synthase, mPGES-1, is an ER target gene that is up-regulated in the breast cancer cell line, MCF-7, following estrogen and cytokine stimulation. An estrogen response element (ERE) was identified in the promoter region of mPGES-1 and subsequent analyses revealed that estradiol stimulated promoter activation. Further, co-treatment with the pro-inflammatory cytokine, TNF-α and estradiol caused a synergistic up-regulation of mPGES-1 expression. Catley et al. (310) implicated a role for NF-κB activation in regulating the cytokine-dependent induction of mPGES-1. In the absence of

estradiol, the data presented by Frasor et al. (209) illustrated that while a IKKβ-dominant negative construct reduced the synergistic up-regulation of mPGES-1 expression by co-treatment with estradiol and TNF-α, no induction of mPGES-1 gene expression was seen with TNF-α alone. This implies that NF-κB is not required for the TNF-α-mediated induction of mPGES-1 and that there may be other elements within the mPGES-1 genome that could potentially contribute to the TNF-α induction.

Results

TNF-α Induces Microsomal PGES-1 Gene Expression in a Time-Dependent and Cell-Specific Manner

In an attempt to elucidate the mechanism of mPGES-1 transcriptional activation following TNF-α treatment in the MCF-7 breast cancer cell line, cells were stimulated with TNF-α. Figure A-1 illustrates that TNF-α increased mPGES-1 mRNA expression about ~9 fold. Alternatively, in HFL-1 cells (normal lung fibroblast cell line) TNF-α stimulated a moderate increase in mPGES-1 levels, about ~4.5 fold. Together the data illustrates that TNF-α can induce mPGES-1 gene expression in a time-dependent and potentially cell-specific manner.

Analysis of the Activation of the Distal Hypersensitive Site (HS2) by TNF-α

The distal hypersensitive site, HS2, in the promoter region of mPGES-1 was recently evaluated for cytokine-induced activation of mPGES-1 gene expression. Therefore, the HS2 fragment (-10.7 to -6.4 kb) driving growth hormone expression was evaluated in MCF-7 cells following induction by TNF-α. The results illustrated in Figure A-2 reveal that wild type promoter activity was not induced by TNF-α, but in the presence of the HS2 fragment, there was an increase in basal expression in the absence of TNF-α followed by a subsequent increase in the induced expression.

Identification of TNF-α Responsive Regulatory Elements within HS2

Sub-fragments of HS2 were previously generated and evaluated for expression in response to another pro-inflammatory cytokine, IL-1β. Due to the TNF-α-mediated induction of the large HS2 fragment, the sub-fragments were next evaluated for TNF-α-responsiveness. The following fragments were evaluated for growth hormone expression: (-10.1 to -9.0 kb), (-8.6 to -6.4 kb), (-8.6 to -8.1 kb) and (-7.6 to -6.4 kb). Figure A-3 reveals that the (-10.1 to -9.0 kb) sub-fragment exhibited an increase in both the basal and induced expression compared to the wild type promoter construct following TNF-α. Further, none of the other fragments which previously showed a significant increase in response to IL-1β treatment responded favorably to TNF-α.

Discussion

In breast cancer versus normal breast tissue, mPGES-1 is known to be highly up-regulated. The steroid hormone, estrogen is known to be active in breast cancer and a literature search revealed a number of studies illustrating a role for estrogen in mPGES-1 gene activation and expression in both a cytokine-dependent and independent manner. Within the mPGES-1 proximal promoter region, an ERE was identified and deemed important for mPGES-1 gene activation following estradiol treatment (209). This estradiol-induction was further enhanced by treatment with the pro-inflammatory cytokine, TNF-α. Alternatively, TNF-α alone, was not able to induce promoter activation and the transcription factor, NFκB was found to have no effect on TNF-α mediated induction of mPGES-1.

In Chapter 4 a distal hypersensitive site, HS2 was identified by DNase I hypersensitive site analysis and it was found to contain IL-1β-responsive element which is required for mPGES-1 gene induction by IL-1β. Therefore, HS2 was evaluated for TNF-α mediated induction of mPGES-1 gene expression. The preliminary data indicates that while the endogenous promoter construct is not activated by TNF-α, the presence of HS2 lead to a significant increase in both

basal and inducible activity following TNF-α treatment. Further analysis of HS2 sub-fragments revealed a potential element within the 5'region of HS2 that is extremely responsive to TNF-α, while analysis of the 3' end of HS2 yielded no significantly active elements. Therefore a finer analysis of the entire HS2 fragment is needed to efficiently delineate the location of the highly responsive basal element and further elucidate the location of an inducible element.

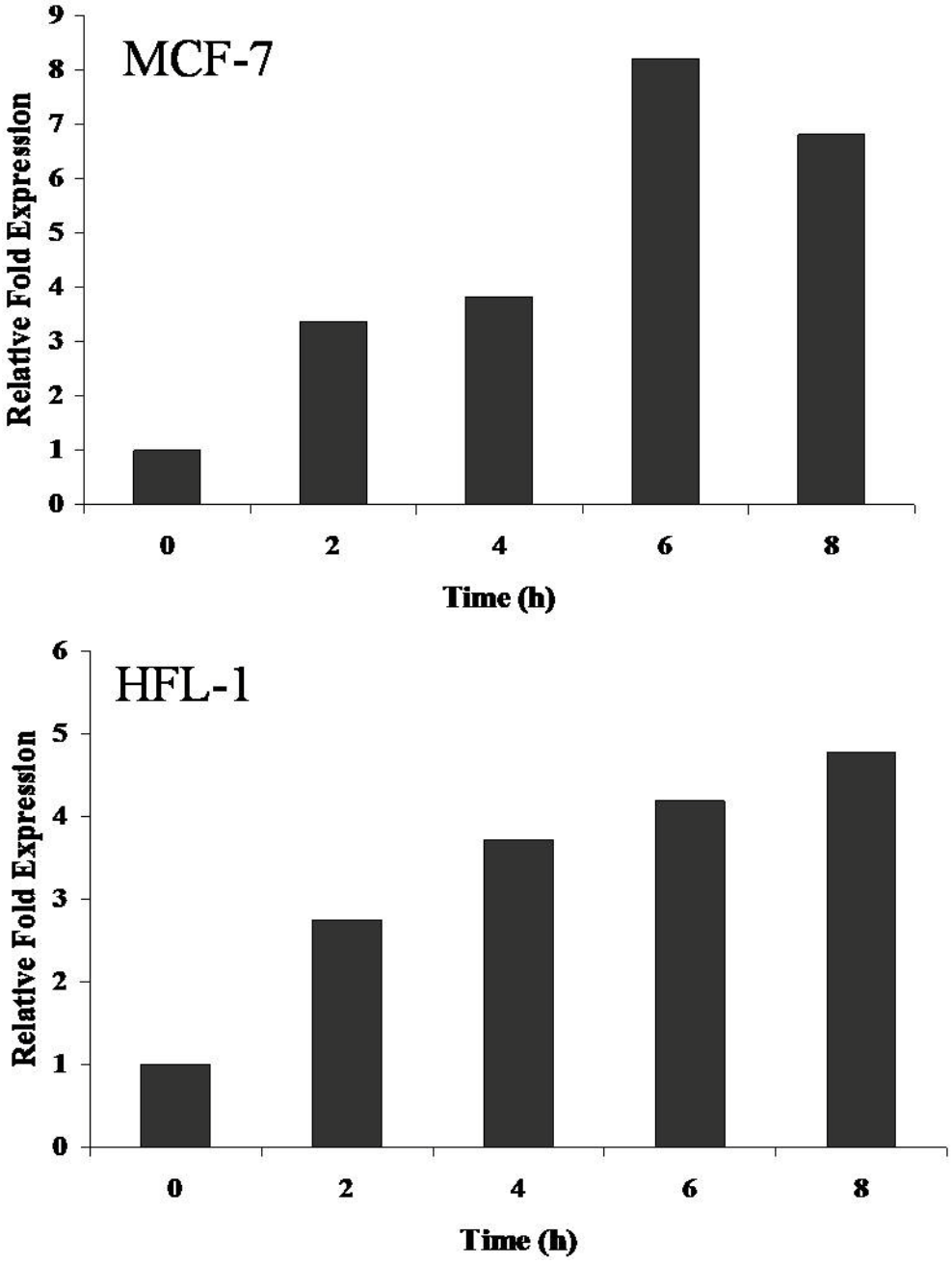

Figure A-1. TNF-α induces mPGES-1 gene expression in a time dependent and cell-specific manner. MCF-7 and HFL-1 cells were stimulated with 10 ng/mL TNF-α, total RNA was isolated and analyzed by real-time RT-PCR.

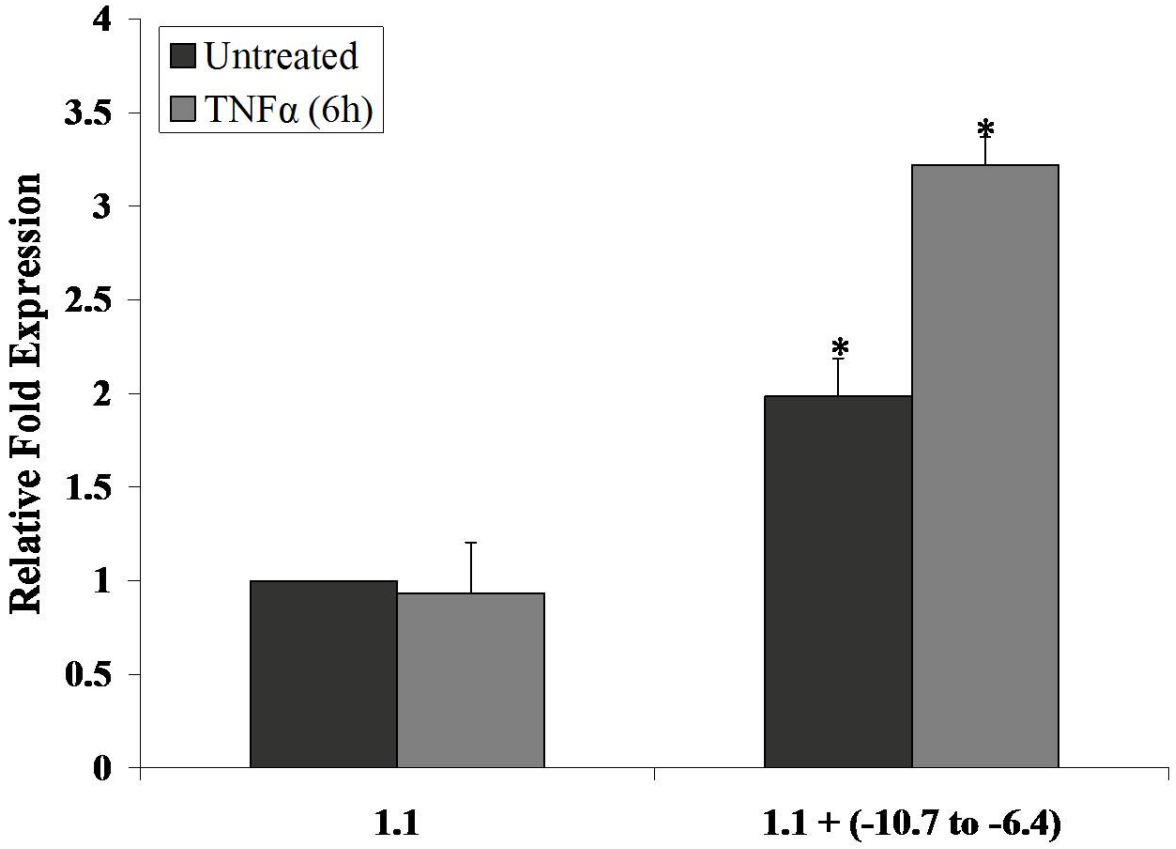

Figure A-2. Analysis of the activation of the distal hypersensitive site (HS2) by TNF-α. MCF-7 cells were transiently transfected with the indicated fragments. 46 h later total RNA was isolated from cells stimulated with or without TNF-α and analyzed by real-time RT-PCR. The mPGES-1/cyclophilin A ratio of untreated cells was set to 1. The graph depicts a summary of three independent experiments, where the data points are represented as mean ± SEM (standard error of the mean). The asterisk (*) indicates statistical significance p value $\leq$ 0.05 as compared with the untreated wild type promoter samples.

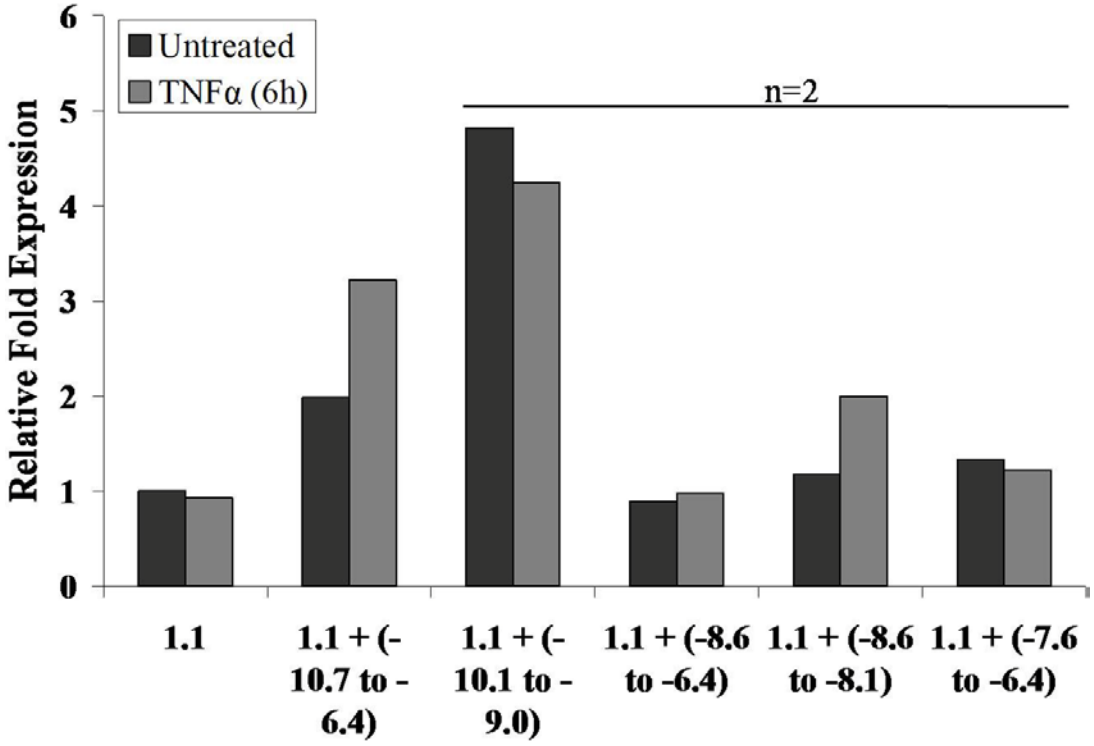

Figure A-3. Identification of TNF-α responsive regulatory elements within HS2. Real-time analysis of MCF-7 cells transiently transfected with the indicated fragments.

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BIOGRAPHICAL SKETCH

Jewell Nadia Walters was born on the island of Tortola in the British Virgin Islands. She attended school and worked there until 1993 when she left to start her undergraduate education at Hampton University in Virginia. After graduating with a Bachelor of Science degree in 2001, she relocated to Maryland and got a job as a research technician at Johns' Hopkins University (Baltimore, Maryland) in the laboratory of Dr. Prashant Desai, working on herpes simplex virus type II. In 2003, she left Johns' Hopkins University and joined the Interdisciplinary Doctoral Program (IDP) at the University of Florida (Gainesville, Florida) and in May 2004, joined Dr. Harry Nick's lab.