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cPLA₂α Gene Activation by IL-1β is Dependent on an Upstream Kinase pathway, Enzymatic Activation and Downstream 15-lipoxygenase Activity: A Positive Feedback Loop

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Abstract

Cytosolic phospholipase A₂α (cPLA₂α) is the most widely studied member of the Group IV PLA₂ family. The enzyme is Ca²⁺-dependent with specificity for phospholipid substrates containing arachidonic acid. As the pinnacle of the arachidonic acid pathway, cPLA₂α has a primary role in the biosynthesis of a diverse family of eicosanoid metabolites, with potent physiological, inflammatory and pathological consequences. cPLA₂α activity is regulated by pro-inflammatory stimuli through pathways involving increased intracellular Ca²⁺ levels, phosphorylation coupled to increased enzymatic activity and *de novo* gene transcription. This study addresses the signal transduction pathways for protein phosphorylation and gene induction following IL-1β stimulation in human fetal lung fibroblasts. Our results utilizing both inhibitors and kinase-deficient cells demonstrate that cPLA₂α is phosphorylated within 10 min of IL-1β treatment, an event requiring p38 MAPK as well as the upstream kinase, MKK3/MKK6. Inhibition of p38 MAPK also blocks the phosphorylation of a downstream, nuclear kinase, MSK-1. Our results further demonstrate that the activities of both cPLA₂α and a downstream lipoxygenase (15-LOX2) are required for IL-1β-dependent induction of cPLA₂α mRNA expression. Overall, these data support an MKK3/MKK6→p38 MAPK→MSK-1→cPLA₂α→15-LOX2-dependent, positive feedback loop where a protein's enzymatic activity is required to regulate its own gene induction by a pro-inflammatory stimulus.

Keywords

phospholipase; cPLA₂α; cytokine; signaling; positive feedback; lipoxygenase

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1. Introduction

Lipid mediators are extremely important messengers with diverse biological activities including central roles in normal physiological responses, inflammation, cell death and cancer [1]. The first step in the formation of these eicosanoids is the cleavage of arachidonic acid from membrane-bound phospholipids by the action of phospholipase A₂s (PLA₂s) [2, 3]. PLA₂s represent an important superfamily of enzymes that catalyze the rate limiting hydrolysis of membrane phospholipids. One of the most widely studied PLA₂s is the calcium-dependent, cytosolic cPLA₂α, which displays a high level of specificity in the cleavage of arachidonic acid from the sn-2 position [4]. This specificity for arachidonic acid and its involvement in the production of downstream eicosanoids has established the central importance of this isozyme in cellular signaling and disease pathology [4]. The significance of cPLA₂α is best illustrated by the pathology of cPLA₂α-deficient mice in disease models of autoimmune diabetes [5], atherogenesis [6], MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) neurotoxicity (Parkinson's disease model) [7], collagen-induced arthritis [8], anaphylaxis [9], acute lung injury [9], and experimental allergic encephalomyelitis (multiple sclerosis) [10].

The regulatory mechanisms underlying the control of cPLA₂α are multifaceted, occurring in minutes, through intracellular calcium release or protein phosphorylation, or within a few hours, as a consequence of stimulus-specific *de novo* transcription. At the protein level, cPLA₂α possesses an N-terminal C2 domain that rapidly responds to stimulus-initiated, micromolar increases in intracellular Ca²⁺ concentrations [11], directing the translocation of cPLA₂α from the cytosol to the nuclear/ER membrane [12]. This is an essential step in the activation of the enzyme because translocation of cPLA₂α to the perinuclear membrane facilitates its proximity to its substrate and coupling to the downstream enzymes in the eicosanoid pathway. Extensive studies have also implicated cPLA₂α phosphorylation as an additional cell type- or stimulus-specific regulatory mechanism that can reportedly increase catalytic activity or influence membrane binding affinity associated with transient increases in intracellular calcium [4, 13]. Three relevant residues, Ser505, Ser515, and Ser727, have been reported as phosphorylation sites through the action of either mitogen-activated protein kinases (MAPKs) [14], mitogen-activated protein kinase interacting kinase (MNK1) [11] or calcium/calmodulin-dependent kinase II (CaMKII) [15]. For example, the serine at position 505 on cPLA₂α has been reported to be phosphorylated by ERK and p38 MAPKs in response to a variety of agonists [16, 17]. Most important to the current studies are the association of specific kinase pathways with IL-1β-dependent regulation of both cPLA₂α phosphorylation and transcriptional activation of cPLA₂α gene expression.

cPLA₂α is basally expressed at low levels in normal cells, and the gene can be transcriptionally activated in response to pro-inflammatory stimuli (IL-1β, TNF, IFN-γ, LPS and zymosan) [18–21], phorbol ester [22], exposure to *Listeria monocytogenes* [23] and various growth factors [24, 25]. This transcriptional activation occurs within a few hours following stimulation, which is preceded by rapid changes associated with intracellular Ca²⁺ increases, protein phosphorylation, translocation, substrate/membrane affinity and increases in enzyme activity. The increased expression of cPLA₂α in response to pro-inflammatory cytokines is a result of *de novo* transcription, as previously shown by our laboratory using nuclear run-on assays [20, 21]. Furthermore, treatment with glucocorticoids or IL-4 has been shown to effectively down-regulate both basal and stimulus-dependent gene expression [26]. To date however, very little is known about the *cis*-acting regulatory elements required for induction of cPLA₂α gene expression by pro-inflammatory cytokines or other stimuli [4]. In an effort to elucidate the intracellular signaling mechanisms that orchestrate IL-1β induction of cPLA₂α in pulmonary cells, we explored potential kinase cascades that mediate the phosphorylation of this enzyme and their role in transcriptional induction.

2. Materials and Methods

2.1. Reagents

FuGENE® 6 transfection reagent and complete protease inhibitor cocktail were purchased from Roche Applied Science. IL-1 β was purchased from R&D Systems, Inc.. AACOCF₃, pyrrolidine, SP600125, PD98059, SB203580 and SB202190 were purchased from Calbiochem. Luteolin, MK-886, NDGA and indomethacin were purchased from Cayman Chemical. PD146176 was obtained from Sigma-Aldrich Corp. siRNAs against 15-LOX2 and luciferase were purchased from Qiagen. 15-LOX2 and cPLA₂ α antibodies were purchased from Oxford Biomedical Research Inc and Santa Cruz Biotechnology, respectively. Phospho-cPLA₂ (Ser505), phospho-MSK1 (Ser376) and phospho-MKK3/MKK6 (Ser189/207) antibodies were purchased from Cell Signaling Technologies. Random Primers DNA Labeling Kit and First Strand Synthesis Kit were purchased from Invitrogen and bicinchoninic acid protein assay kit from Pierce.

2.2. Cell Culture

Human Fetal Lung Fibroblast (HFL-1) cells from ATCC were grown in Ham's F12K medium (Sigma-Aldrich Corp) supplemented with 10% fetal bovine serum, 4 mM glutamine, ABAM (10 μ g/mL penicillin G, 0.1 mg/mL streptomycin, and 0.25 μ g/mL amphotericin B) (Invitrogen) at 37°C in humidified air with 5% CO₂. Mouse embryonic fibroblasts (MEF) (wild type and p38 α -/-) were kindly provided by Dr. A. Nebreda (EMBL), (wild type and p38 β -/-) were kindly provided by Dr. A. Choi (Harvard Medical School) and (wild type and MKK3/6 -/-) were kindly provided by Dr. R. Davis (University of Massachusetts). All MEF cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum and ABAM (10 μ g/mL penicillin G, 0.1 mg/mL streptomycin, and 0.25 μ g/mL amphotericin B) in continuous culture at 37 °C in humidified air with 5% CO₂. Experimentally, cells were grown to 70–75% confluency before treatment with pharmacologic agents, and/or cytokines. Cells were treated with all pharmacologic inhibitors for 1 h prior to exposure to IL-1 β at 2 ng/mL.

2.3. RNA Isolation, Northern Analysis and Real-time RT-PCR

20 μ g of total cellular RNA was isolated and size-fractionated on a 1% agarose-formaldehyde gel as previously described [20]. The size fractionated RNA was electrotransferred to a nylon membrane (Zeta-Probe membrane, Bio-Rad, Hercules, CA) and UV cross-linked. The membrane was hybridized with randomly primed double stranded ³²P-labeled probes for human cPLA₂ α or human large subunit ribosomal L7a overnight as previously described [20] followed by exposure to X-ray film. cDNA was synthesized with First Strand Synthesis Kit (Invitrogen) and analyzed by real-time PCR analysis using an ABI 7000 Sequence Detection System. The relative fold expression, normalized to cyclophilin A, was determined by the $\Delta\Delta$ CT method as described [27].

2.4. Immunoblot Analysis

Total cellular lysates were isolated by homogenization in RIPA buffer (50 mM HEPES pH 7.3, 150 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 1% Triton X-100, 10 % glycerol, 1 mM DTT and protease inhibitor cocktail) with a hand held homogenizer. Lysates were centrifuged at 14,000 g for 15 min at 4°C to remove cellular debris. The supernatant was transferred to pre-chilled 1.5 mL microcentrifuge tubes and total protein was determined by modified Bradford assay kit. 30 to 50 μ g of protein were loaded onto 4–20% Tris-HCl Criterion gels and run for approximately 2 h at 100 V then electrotransferred to nitrocellulose membranes overnight at 30 V at 4°C. Membranes were subsequently blocked with 7.5% non-fat dry milk or 7% BSA in TBS with 0.1% Tween (TBST) overnight at 4°C.

Blots were washed three times in 1X TBST for 5 min, incubated with primary antibodies in 7% BSA/TBST either at room temperature for 1–4 h or overnight at 4°C. Following primary antibody incubation, blots were washed as previously stated and incubated with secondary antibodies in 7% BSA/TBST for 1 h at room temperature, followed by washes with TBST. An enhanced chemiluminescence kit (Amersham) was used for detection.

2.5. siRNA analysis

HFL-1 cells were transfected with a final concentration of 100 nM 15-LOX2 siRNA (Qiagen) using HiPerfect transfection reagent (Qiagen) according to the manufacturer's recommendation. As a control for off-target effects of siRNA, cells were also transfected with a luciferase-specific siRNA (Qiagen) and designated as control/nonspecific siRNA. 48 h post transfection cells were rinsed with PBS and incubated with fresh media plus siRNA. At 96 h post transfection, cells were treated with or without IL-1 β (2 ng/mL).

2.6. Densitometry and Statistical Analyses

Densitometry was quantified from autoradiography films using a Microtek scan maker 9600XL and analyzed with NIH ImageJ software. Statistical analyses were performed by a Student's t-test and * denotes $p < 0.05$ while ** denotes $p < 0.01$.

3. Results

3.1. IL-1 β -dependent phosphorylation and transcriptional activation of cPLA $_2\alpha$ requires p38 MAPK activity

Numerous stimuli, including colony stimulating factor 1 [25], immunoglobulin E Fc receptor [28], thrombin [29], zymosan, phorbol esters [30] and IL-1 β [13], can induce phosphorylation of cPLA $_2\alpha$ leading to the rapid concomitant increase of cPLA $_2\alpha$ enzymatic activity. For all stimuli, the induction of cPLA $_2\alpha$ enzymatic activity occurs within minutes and is marked by a rapid release of its substrate, arachidonic acid, along with an increase in the affinity for membrane phospholipids in a calcium-dependent manner. Phosphorylation of cPLA $_2\alpha$ at Ser505 has been implicated in this process [17, 31, 32] and substantiated by overexpression of a cPLA $_2\alpha$ mutant, Ser505Ala, exhibiting no enhanced agonist-stimulated arachidonic acid release [17]. We have previously demonstrated a dose- and time-dependent induction of human cPLA $_2\alpha$ mRNA and protein by IL-1 β , TNF- α , and IFN- γ in human lung epithelial and fibroblast cells that is dependent on *de novo* transcription, with induction occurring within 3 hours [20]. In order to understand the intracellular signaling pathways involved in IL-1 β stimulation, a human fetal lung fibroblast cell line, HFL-1, was exposed to IL-1 β for increasing durations up to 60 min and evaluated by immunoblot analysis with a phospho-specific antibody to Ser505 of cPLA $_2\alpha$ with corresponding densitometry (Fig. 1A). Phosphorylation of cPLA $_2\alpha$ occurs within 10 min and maximal levels are achieved by 1 h. Alternatively, treatment with an inhibitor of p38 MAPK, SB203580, completely blocked the IL-1 β -dependent cPLA $_2\alpha$ phosphorylation.

There have been varied reports on the specific kinases responsible for the phosphorylation of cPLA $_2\alpha$ [14, 16, 33–35]. To correlate the rapid phosphorylation event with transcriptional induction and demonstrate the specificity of p38 MAPK, we examined the effects of inhibitors for p38 MAPK (SB203580 and SB202190), ERK1/2 (PD98059) and JNK (SP600125) (Fig. 1B, top). The results illustrate that inhibition of p38 MAPK alone blocked the IL-1 β -dependent induction of cPLA $_2\alpha$ gene expression. Similarly, cPLA $_2\alpha$ protein levels following IL-1 β treatment were inhibited only by a p38 MAPK inhibitor (Fig. 1B, bottom). To further demonstrate the efficacy of SB203580 on IL-1 β -dependent induction, HFL-1 cells were treated with concentrations up to 10 μ M as per the dose used in Figure 1B.

Densitometry of the resulting northern analyses showed a > 50% inhibition at 0.5 μ M (Fig. 1C).

Of the four known p38 isoforms, p38 α , p38 β , p38 γ and p38 δ , we evaluated two isoforms, p38 α and p38 β , which have been most prominently studied to date [36, 37]. To further verify the involvement of p38 MAPK, mouse embryonic fibroblasts (MEF) deficient in p38 α or p38 β were analyzed for cPLA $_2\alpha$ gene induction by IL-1 β . The real-time RT-PCR results demonstrate that cPLA $_2\alpha$ expression is induced in wild type MEFs (+/+) whereas knockout MEFs for either p38 α (-/-) or p38 β (-/-) exhibited no induction (Fig. 1D).

3.2. IL-1 β -dependent induction of cPLA $_2\alpha$ involves a MKK/p38/MSK-1 cascade

Studies evaluating phosphorylation of cPLA $_2\alpha$ have been limited to addressing the involvement of MAPKs or other individual kinases, while no studies have attempted to link individual signaling events with respective upstream or downstream pathways [11]. Increasing evidence has connected the upstream activation of p38 MAPK through the involvement of mitogen-activated protein kinase kinase, MKK, specifically MKK3 and MKK6 [38, 39]. Immunoblot analysis and corresponding densitometry of HFL-1 cells exposed to IL-1 β using a phospho-specific antibody that recognizes both Ser189 and 207 from MKK3 and MKK6, respectively, demonstrated that IL-1 β causes a rapid phosphorylation of these kinases (Fig. 2A). This provides a potential direct connection with the IL-1 receptor complex through the upstream kinase, MAPK-ERK kinase kinase (MEKK3), which is known to activate MKK3 [36], and has been shown to be an important transducer of the MyD88-IRAK-TRAF6 complex in IL-1R-TLR4 signaling [40]. To further illustrate the role of MKK3/MKK6 in p38 MAPK activation and, potentially, cPLA $_2\alpha$ gene expression, wild type (+/+) and MKK3/MKK6 deficient MEFs (-/-) were evaluated for cPLA $_2\alpha$ expression (Fig. 2B). These data demonstrate that IL-1 β induced cPLA $_2\alpha$ expression in wild type MEFs but not in the MKK3/MKK6 deficient MEFs.

We next attempted to address the signaling pathway that may lie downstream of p38 MAPK. Mitogen- and stress-response kinase 1 (MSK1), and the related kinase MSK2, are nuclear kinases which have been shown to be activated downstream of p38 MAPK [41]. Two independent groups have connected MSK activity with cPLA $_2\alpha$, where Markou et al. [42] showed that purinergic stimulation causes both MSK1 phosphorylation and cPLA $_2\alpha$ translocation, and Börsch-Haubold et al. [43] implicate MSK1 as the downstream kinase in agonist stimulated platelets which phosphorylates Ser727 in cPLA $_2\alpha$ leading to increased arachidonate release. Immunoblot analysis and corresponding densitometry illustrates a time-dependent phosphorylation of MSK-1 at Ser176 by IL-1 β that can be blocked by the p38 MAPK inhibitor, SB203580 (Fig. 2C). To date, no specific inhibitors of MSK have been identified, and attempts to procure MSK1/MSK2 deficient MEF cells were not successful.

3.3. IL-1 β -dependent transcriptional induction of cPLA $_2\alpha$ requires a positive metabolic feedback loop

We next hypothesized that activation of cPLA $_2\alpha$ enzymatic activity may in part be responsible for the concomitant increase in its own gene expression. The involvement of cPLA $_2\alpha$ in the control of intracellular downstream events has been previously highlighted, for example, in the control of TNF-induced cell death [44], the TNF-dependent nuclear translocation of NF- κ B and NF- κ B-activated gene expression [45], the cardiac beta2-AR signaling pathway [46], and gene expression for type IIA secretory phospholipase A $_2$ [47], IL-8 and COX-2 [48]. These studies were based, in part, on the inhibition of cPLA $_2\alpha$ enzymatic activity by competitive inhibitors such as arachidonyl trifluoromethyl ketone (AACOCF $_3$) [49]. Based on the timing of the phosphorylation and transcriptional induction

of cPLA₂ α , we exposed cells to increasing concentrations of AACOCF₃ alone or in the presence of IL-1 β and evaluated the results by northern analysis (Fig. 3A). As shown, inhibition of cPLA₂ α enzymatic activity caused a dose-dependent reduction in the level of induced cPLA₂ α mRNA with an almost 80% inhibition at 50 μ M. To further validate the importance of cPLA₂ α enzymatic activity on transcriptional induction, we also treated cells with pyrrolidine [50], another purported inhibitor of cPLA₂ α , alone or in combination with IL-1 β . As observed with AACOCF₃, 4 μ M pyrrolidine caused a ~50% inhibition of the IL-1 β -dependent induction of cPLA₂ α mRNA levels (Fig. 3B), thus implicating arachidonic acid and possibly its downstream eicosanoid metabolites in a positive feedback regulatory pathway.

Following arachidonic acid liberation from membrane phospholipids by cPLA₂ α , it is further metabolized through either the cyclooxygenase or lipoxygenase pathways. To address the importance of downstream events on the IL-1 β induction of cPLA₂ α , we next evaluated indomethacin, a nonselective cyclooxygenase inhibitor with no effect (data not shown). In contrast, inhibition of leukotriene production with a non-selective lipoxygenase (LOX) inhibitor, nordihydroguaiaretic acid (NDGA), demonstrated a ~85% inhibition of IL-1 β -dependent cPLA₂ α transcription at 50 μ M (Fig. 4A). NDGA has been shown to inhibit 5, 12- and 15-LOXs with only slightly different IC₅₀s in the low to mid μ M range. To determine which LOX enzymes may be involved in transcriptional induction, we utilized a variety of other LOX inhibitors. Among the LOXs, 5-LOX catalyzes the first step in the oxygenation of arachidonic acid producing 5-hydroperoxyeicosatetraenoic acid (5-HPETE). 5-LOX requires 5-LOX-activating protein (FLAP) to become catalytically active [51]. Cells were treated with MK-886, which binds to FLAP with high-affinity and prevents 5-LOX activation. The representative experiment in Figure 4B illustrates that this inhibitor has no effect on the induction of cPLA₂ α by IL-1 β . Similarly, we also treated HFL-1 cells with baicalein, a reported inhibitor of platelet 12-lipoxygenase [52] with no effect (data not shown).

We next evaluated a plant flavanoid, luteolin, which has been alleged to have a high level of selectivity towards 15-LOX [53]. It should be noted that this family of compounds has also been reported to inhibit tyrosine kinase activity [54]; however, a close examination of many of the physiological consequences, such as inhibition of histamine release, CD40 ligand expression and synthesis of IL-4 and IL-13 [55], are highly consistent with their ability to effectively inhibit 15-LOX activity [53]. In HFL-1 cells exposed to luteolin alone or in combination with IL-1 β , we observed a ~50% inhibition at 10 μ M and almost complete block of IL-1 β induction of cPLA₂ α mRNA levels at 50 μ M (Fig. 5A).

15-LOX has been described as a catalyst of enzymatic lipid peroxidation with the ability to execute oxidative modifications such as the oxidation of LDL *in vitro* as well as through overexpression in cells and transgenic animal models [56]. As a consequence of the enzyme's general oxidative activity, many of the known LOX inhibitors, such as PD146176 [57, 58], have also been purported to possess general anti-oxidant activities. To this end, Sendobry et al. [59] identified a compound, PD146176, which lacked non-specific antioxidant activity but was reported to specifically inhibit 15-LOX with moderate inhibitory activity against 5- or 12-LOX. More recently, mice chronically treated with PD146176 exhibited significantly worse intestinal function during experimental colitis relative to untreated mice [60], demonstrating the *in vivo* effectiveness of this inhibitor. Therefore, we treated HFL-1 cells with PD146176 and found that this 15-LOX inhibitor, similarly to NDGA and luteolin, was able to effectively inhibit induction of cPLA₂ α mRNA by IL-1 β (Fig. 5B).

Since there are two isoforms of 15-LOX, 15-LOX1 and 15-LOX2, we first verified the expression of each gene in our cells by real-time RT-PCR and found that only 15-LOX2 was basally expressed in HFL-1 cells. Therefore, in an attempt to further highlight the importance of 15-LOX in mediating the IL-1 β induction of cPLA $_2\alpha$ gene expression, we sought to confirm the effects of the enzymatic inhibitors by employing specific knockdown of 15-LOX2 expression by siRNA. HFL-1 cells were transfected with an siRNA against 15-LOX2 as compared to a non-specific siRNA and cPLA $_2\alpha$ mRNA levels were analyzed by real-time RT-PCR following IL-1 β treatment. The immunoblot in Figure 5C illustrates that the siRNA specifically reduced 15-LOX2 protein expression, while the real-time RT-PCR data (Fig. 5C) illustrate that siRNA to 15-LOX2 caused a ~50% reduction in IL-1 β -dependent cPLA $_2\alpha$ gene expression providing evidence for a positive feedback regulatory mechanism. Attempts to mimic the involvement of 15-LOX2 utilizing either extracellular exposure to arachidonic acid or 15S-hydroxyeicosatetraenoic acid (15-HETEs) treatment of HFL-1 cells did not show any level of mRNA induction.

4. Discussion

As the pinnacle of the eicosanoid pathway, cPLA $_2\alpha$ activity is essential for initiating the release of arachidonic acid for the production of bioactive downstream eicosanoids. Studies utilizing cPLA $_2\alpha$ deficient mice have provided insight as to the role of this enzyme in diverse physiological and pathological events. For example, these animals exhibit decreased allergic airway responsiveness [61] as well as defects in female reproduction [62] and renal physiology [63]. The results of these and other studies illustrate a central role for cPLA $_2\alpha$ and possibly the downstream arachidonic acid metabolites in the regulation of both physiological and pathological events.

Numerous studies have demonstrated that cPLA $_2\alpha$ enzymatic activation is a consequence of phosphorylation events and increases in intracellular calcium levels [13, 25, 28–30]. In fact, Kramer et al. [29] demonstrated that treatment human platelets with the agonist, thrombin, leads to phosphorylation of cPLA $_2\alpha$ and increased enzymatic activity on a minute time scale. In a similar study, Qiu et al. [30] also showed increased cPLA $_2\alpha$ enzymatic activity as a consequence of phosphorylation in mouse peritoneal macrophages stimulated with zymosan. Gronich et al. [13] reported that rat mesangial cells exposed to IL-1 α stimulated the phosphorylation of cPLA $_2\alpha$, leading to a rapid increase in enzymatic activity and subsequent arachidonic acid release. These studies support the importance of post-translational modifications on the rapid enzymatic activation of cPLA $_2\alpha$.

Our results in IL-1 β -stimulated human lung fibroblasts link a specific kinase pathway leading to cPLA $_2\alpha$ phosphorylation coupled with increased enzymatic activity, culminating in *de novo* induction of cPLA $_2\alpha$ gene transcription. The results support a p38 MAPK centered pathway implicating both upstream (MKK3/MMK6) and downstream kinase (MSK1) events to the ultimate phosphorylation of cPLA $_2\alpha$. The use of pharmacological inhibitors of p38 MAPK in conjunction with mouse embryonic fibroblasts deficient for either the two major p38 MAPK isoforms or MKK3/MKK6 demonstrate the involvement of a specific p38 MAPK cascade in the induction of cPLA $_2\alpha$. Furthermore, one might also implicate IL-1 β signaling through the MyD88-IRAK-TRAF6/IL-1R-TLR4 receptor complex with the documented activation of the MAPK-ERK kinase kinase (MEKK3) which has been linked to downstream MKK3 activation [36, 40, 64]. Interestingly, a comparison of the time-dependent phosphorylation events in (Figs. 1A and 2A), where the maximal level of phosphorylation occurs for MKK3/MKK6 within 10 min, culminating in maximal p38 MAPK-dependent cPLA $_2\alpha$ phosphorylation by 20 min. This would therefore support a temporal sequence of events, namely MKK3/MKK6 $\rightarrow$ p38 MAPK $\rightarrow$ cPLA $_2\alpha$. Our results also implicate the rapid IL-1 β -induced phosphorylation of the nuclear kinase, MSK-1, in a

p38-dependent manner, in the induction of cPLA₂α mRNA and protein levels following IL-1β stimulation (Fig. 2C). Although we were not able to obtain MSK-1 deficient mouse embryonic fibroblasts to illustrate the specific involvement of MSK-1 in the induction of cPLA₂α gene expression, other studies have indicated that MSK-1 may be responsible for the phosphorylation of cPLA₂α [42, 43]. In addition, the maximal phosphorylation of MSK-1 (~20 min) also supports the temporal sequence of events on the minutes time scale summarized in Figure 6.

Given the connection of increased phosphorylation and enzymatic activity [13, 25, 28–30], our results further associate cPLA₂α enzymatic activity with the subsequent induction of cPLA₂α mRNA levels by IL-1β (Fig. 3). Furthermore, we have demonstrated the importance of the lipoxygenase pathway, specifically the activity/product of 15-LOX2 (Figs. 4 and 5), through enzymatic inhibition and siRNA-mediated knockdown. The timing of transcriptional activation is supported by our previous studies which demonstrate *de novo* transcription within 3 hours based on nuclear run-on analysis [20]. As shown on the right side of Figure 6, we believe that, subsequent to the phosphorylation of cPLA₂α on the minute time scale, the activation of gene expression through 15-LOX2 then occurs within the first few hours of IL-1β stimulation. While extracellular 15-HETE has been previously utilized to induce gene activation of peroxisome proliferator-activated receptor γ in prostate carcinoma cells [65], we were unable to recapitulate the IL-1β induction with exogenous arachidonic acid or 15-HETE treatment. At this time, we have not identified the precise role of 15-LOX2 activity or potential downstream products in the transcriptional activation of cPLA₂α. Of possible importance, 15-LOX2 has been shown to function as a tumor suppressor and/or negative cell cycle regulator in the prostate [66], however as with our results the precise mode of action has yet to be elucidated.

Our results, as summarized in Figure 6, therefore establish prerequisites for IL-1β-mediated cPLA₂α gene activation which link protein phosphorylation/enzyme activity with downstream 15-LOX2 activity/products. This sequence of events can be explained by a positive feedback loop [67]. However our results also require an autoregulatory event within the positive feedback mechanism, in that initial rapid increases in cPLA₂α phosphorylation/activation ultimately lead to increases in its own gene transcription, feeding back through subsequent protein synthesis to complete the cycle. This result fulfills the description of a positive feedback loop where the end of a sequence perpetuates the initial propagating event. Our current efforts are focused on determining specific effects of 15-LOX2 as well as pursuing the location of DNA regulatory elements and their cognate regulatory factors which orchestrate IL-1β-mediated cPLA₂α gene regulation.

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Abbreviations

TRAF6	TNF receptor-associated factor 6
MyD88	Myeloid differentiation factor 88
IRAK	Interleukin-1 receptor associated kinase
MEKK3	MAP kinase kinase kinase 3
MKK	mitogen activated protein kinase kinase

MSK-1 mitogen and stress activated protein kinase 1
LOX lipoxygenase

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Figure 1. IL-1 β -dependent induction of cPLA $_2\alpha$ requires p38 MAPK for both protein phosphorylation and transcriptional activation

A) HFL-1 cells were pre-treated with or without the p38 MAPK inhibitor, SB203580 (SB203, 10 μ M) for 1 h followed by exposure to IL-1 β for the indicated times. Phospho-cPLA $_2\alpha$ (Ser505) levels were analyzed by immunoblot analysis. The bar graph summarizes densitometric data as mean values $\pm$ SEM (n=3). * denotes significance of $p < 0.05$ and ** signifies $p < 0.01$ as compared to untreated cells. **B)** HFL-1 cells were untreated (Control, C) or pre-treated for 1 h with inhibitors for p38 MAPK (SB203580, 10 μ M (SB203) or SB202190, 20 μ M (SB202)), JNK (SP600125, 50 μ M (SP)), ERK (PD98059, 50 μ M (PD)), or the vehicle (dimethyl sulfoxide (DMSO, D)) followed by IL-1 β for 8 h. cPLA $_2\alpha$ mRNA was evaluated by northern analysis (top) and membranes re-probed for a ribosomal protein, L7a, as an internal control. cPLA $_2\alpha$ protein expression was also studied by immunoblot analysis (bottom) from similarly treated cells. **C)** HFL-1 cells were pre-treated for 1 h with increasing concentrations of SB203580 alone or in combination with IL-1 β for 8 h. cPLA $_2\alpha$ and L7a (loading control) were analyzed by northern analysis with corresponding densitometry data. IL-1 β treatment alone is designated as 100%. The number in parentheses above each point designates the number of independent data points. * denotes significance of $p < 0.05$ and ** denotes $p < 0.01$ as compared to IL-1 β alone. **D)** Wild type (+/+) and knockout (-/-) mouse embryonic fibroblasts for p38 α and p38 β MAPK were stimulated with IL-1 β for 8 h. Total RNA was subjected to real-time RT-PCR analysis, and cPLA $_2\alpha$ levels of untreated cells were set to 1. Data points are represented as mean of $2^{-\Delta\Delta CT} \pm$ SEM (n=3). ** denotes significance of $p < 0.01$ as compared to the respective untreated samples.

Figure 2. The IL-1 β -dependent induction of cPLA $_2\alpha$ involves an MKK3/MKK6/p38 MAPK/MSK-1 cascade

A) Immunoblot analysis of phospho-MKK3/MKK6 (Ser189/207) from cells treated with IL-1 β for 0–60 min. The bar graph summarizes densitometry data as means $\pm$ SEM (n=3). * denotes significance of $p < 0.05$ compared to untreated cells. **B)** Wild type (+/+) and MKK3/6 (-/-) mouse embryonic fibroblasts were stimulated with IL-1 β for 8 h and analyzed by real-time RT-PCR analysis with cPLA $_2\alpha$ levels for untreated cells set to 1. Values are represented as a mean of $2^{-\Delta\Delta CT} \pm$ SEM (n=3), and * denotes significance of $p < 0.05$ as compared to respective untreated cells. **C)** Immunoblot analysis of phospho-MSK-1 from cells treated with IL-1 β for 0–60 min. The arrowhead ($\blacktriangleright$) indicates nonspecific bands with the arrow ($\rightarrow$) denoting the ~90 kDa band for phospho-MSK-1. The bar graph summarizes densitometry data and represents means $\pm$ SEM (n=3). * denotes significance of $p < 0.05$ and ** denotes $p < 0.01$ as compared to IL-1 β alone.

Figure 3. cPLA₂α enzymatic activity is required for IL-1β-dependent induction of its own mRNA

A) Northern analysis of cPLA₂α and the loading control, L7a, from control (C) and cells pre-treated with vehicle (E, ethanol) or increasing doses of the cPLA₂α enzymatic inhibitor, arachidonyl trifluoromethyl ketone (AACOCF₃). Densitometry data were plotted as mean ± SEM (n=3) with * denoting significance of p 0.05 and ** denoting p 0.01 as compared to IL-1β alone. **B)** HFL-1 cells were pre-treated in a dose-dependent manner with another cPLA₂α enzymatic inhibitor, pyrrolidine, in the absence or presence of IL-1β. Total RNA was subjected to real-time RT-PCR analysis, and cPLA₂α levels of IL-1β-treated cells were set to 100%. Data points are represented as mean of $2^{-\Delta\Delta CT} \pm SEM$ (n=3). * denotes a value of p 0.05 as compared to the respective untreated samples.

Figure 4. IL-1β-dependent cPLA₂α gene activation requires lipoxygenase activity

A) Northern analysis of cPLA₂α and the loading control, L7a, from cells pre-exposed to the non-selective lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA) at the indicated concentrations for 1 h and then stimulated with or without IL-1β for 8 h. The corresponding graph summarizes densitometry and data points represent means ± SEM (n=3). * denotes significance of p < 0.05 and ** denotes p < 0.01 as compared to IL-1β alone. **B)** A representative northern analysis of cPLA₂α and the loading control, L7a, from cells pre-treated in a dose-dependent manner with the FLAP/5-lipoxygenase inhibitor, MK-886, in the absence or presence of IL-1β.

Figure 5. Inhibition of 15-LOX activity blocks IL-1 β -dependent induction of cPLA₂ α gene expression

A) Northern analysis of cPLA₂ α and the loading control, L7a, from cells pre-exposed to the vehicle, DMSO (D), or increasing concentrations of the 15-lipoxygenase inhibitor, luteolin, followed by exposure to IL-1 β for 8 h. The corresponding graph summarizes densitometry and data points represent means $\pm$ SEM (n=3). ** denotes p < 0.01 as compared to IL-1 β alone. **B)** Northern analysis of cPLA₂ α and the loading control, L7a, from cells pre-treated for 1 h with DMSO (D) or increasing concentrations of the 15-LOX inhibitor, PD146176 (PD146) alone or followed by stimulation with IL-1 β for 8 h. The graph summarizes densitometry data, where values are represented as mean $\pm$ SEM (n=2 at 1 and 10 μ M, n=3

at 100 μ M). * denotes significance of $p \leq 0.05$ and ** denotes $p \leq 0.01$ as compared to IL-1 β alone. C) HFL-1 cells were transfected with a control luciferase siRNA or an siRNA against human 15-LOX2, with or without 4 h IL-1 β and analyzed by immunoblot analysis (top). The lane labeled as "No Tx" refers to untransfected cells. Additionally, total RNA was subjected to real-time RT-PCR analysis of cPLA $_2\alpha$ mRNA with untreated cells set to 1. The graph (bottom) represents real-time RT-PCR data with values represented as the mean of $2^{-\Delta\Delta CT} \pm \text{SEM}$ (n=3). * denotes significance of $p \leq 0.05$ as compared to "No Transfection" or "siRNA Nonspecific."

Figure 6. Schematic of IL-1 β -dependent induction of cPLA $_2\alpha$ gene expression: a Positive Feedback Loop

The left side of the figure illustrates the kinase cascade leading to the IL-1 β -dependent phosphorylation of cPLA $_2\alpha$ occurring on a minute timescale. The “p” designates the series of phosphorylation intermediates. The asterisk (*) indicates that previously reported studies have shown that MSK-1 can phosphorylate cPLA $_2\alpha$ while numerous other studies have demonstrated that stimulus-specific phosphorylation of cPLA $_2\alpha$ leads to increased enzyme activity ultimately resulting in elevated levels of arachidonic acid. The right side of the figure illustrates the action of 15-LOX2 on cPLA $_2\alpha$ -derived arachidonic acid leading to increased cPLA $_2\alpha$ gene expression by an as of yet unknown mechanism (?). This portion of the pathway occurs within hours of initial stimulation. The positive feedback loop is completed by the subsequent increase in cPLA $_2\alpha$ protein levels.