Cytosolic Phospholipase A$_2$ Group IV$\alpha$ but Not Secreted Phospholipase A$_2$ Group IIA, V, or X Induces Interleukin-8 and Cyclooxygenase-2 Gene and Protein Expression through Peroxisome Proliferator-activated Receptors $\gamma$ 1 and 2 in Human Lung Cells*

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It has been reported that interleukin-8 (IL-8) and cyclooxygenase-2 (COX-2) expression is regulated by peroxisome proliferator-activated receptor (PPAR)-$\gamma$ synthetic ligands. We have shown previously that cytosolic phospholipase A$_2$ (cPLA$_2$) is able to activate gene expression through PPAR-$\gamma$ response elements (Pawliczak, R., Han, C., Huang, X. L., Demetris, A. J., Shelhamer, J. H., and Wu, T. (2002) J. Biol. Chem. 277, 33153–33163). In this study we investigated the influence of cPLA$_2$ and secreted phospholipase A$_2$ (sPLA$_2$) Group IIA, Group V, and Group X on IL-8 and COX-2 expression in human lung epithelial cells (A549 cells). We also studied the results of cPLA$_2$ activation by epidermal growth factor (EGF) and calcium ionophore (A23187) on IL-8 and COX-2 reporter gene activity, mRNA level, and protein synthesis. cPLA$_2$ overexpression and activation increased both IL-8 and COX-2 reporter gene activity. Overexpression and activation of Group IIA, Group V, or Group X sPLA$_2$s did not increase IL-8 and COX-2 reporter gene activity. Methyl arachidonyl fluorophosphate, a cPLA$_2$ inhibitor, inhibited the effect of A23187 and of EGF on both IL-8 and COX-2 reporter gene activity, steady state levels of IL-8 and COX-2 mRNA, and IL-8 and COX-2 protein expression. Small inhibitory RNAs directed against PPAR-$\gamma$1 and -$\gamma$2 blunted the effect of A23187 and of EGF on IL-8 and COX-2 protein expression. Moreover small inhibitory RNAs directed against cPLA$_2$ decreased the effect of A23187 and EGF on IL-8 and COX-2 protein expression. These results demonstrate that cPLA$_2$ has an influence on IL-8 and COX 2 gene and protein expression at least in part through PPAR-$\gamma$.

85-kDa cytosolic phospholipase A$_2$ (cPLA$_2$)$^3$ is a cytoplasmic enzyme that metabolizes phospholipids to release arachidonic acid. Upon activation by various stimuli (including but not limited to calcium ionophore (A23187), IL-1$\beta$, tumor necrosis factor-$\alpha$, and interferon-$\gamma$) cPLA$_2$ is translocated to the cellular membranes, releasing arachidonic acid from membrane phospholipids (1–4). Stimuli such as epidermal growth factor (EGF), oxidative stress, or IL-1$\beta$ may also cause cPLA$_2$ activation through its phosphorylation (5, 6). Previously we have reported that cPLA$_2$ is able to alter gene expression through PPAR-$\gamma$ activation and binding to peroxisome proliferator response elements (4). The calcium ionophore A23187 activates cPLA$_2$ by increasing intracellular calcium levels, causing cPLA$_2$ translocation to the nuclear envelope (4, 6, 7). EGF is also thought to increase cytosolic phospholipase A$_2$ activity through activation of the mitogen-activated protein kinase pathway and phosphorylation of cPLA$_2$ at serine 505 (and possibly other serine residues) (5). Both stimuli are known to increase cPLA$_2$ enzymatic activity and arachidonate release.

PLA$_2$ enzymes catalyze the hydrolysis of membrane glycerophospholipids to liberate arachidonic acid and lysophospholipids. So far, more than 20 enzymes that possess PLA$_2$ activity have been identified and cloned in mammals (for reviews, see Refs. 8–15). PLA$_2$s have been classified according to their molecular weight, homology, and calcium influence on enzyme activity. cPLA$_2$ appears to play a role in intracellular arachidonate release, whereas low molecular weight PLA$_2$s (such as Groups IA, IB, IIA, IIC, and V) may be involved in extracellular arachidonic acid release due to the fact that they are secreted into extracellular milieu upon cell stimulation. Thus, there may be distinct roles or cross-talk between PLA$_2$s in cell signaling (16, 17). On the other hand a group of calcium-independent phospholipases A$_2$ seems to have a broader substrate specificity. Several lines of evidence (for a review, see Ref. 18) suggest that the Group VI iPLA$_2$ may be responsible for phospholipid fatty acid remodeling in resting cells. The role of iPLA$_2$ in intracellular cell signaling remains to be clarified.

IL-8 is a chemokine that is produced and secreted by human lung cells (19, 20). It has strong chemotactic properties for neutrophils and eosinophils. Moreover IL-8 is an important proinflammatory cytokine that plays a role in allergic inflammation.

The IL-8 promoter contains a PPAR-$\gamma$ response element localized to −1060 relative to the transcription start site, suggesting that PPAR-$\gamma$ may play a role in regulation of IL-8 expression through PPAR-$\gamma$ response elements.
transcription. It has been reported that several PPAR-γ agonists such as troglitazone, resiglitazone, and others activate IL-8 transcription and enhanced IL-8 secretion in many cell systems including but not limited to lung cells (21–25).

Cyclooxygenase is a key enzyme in prostaglandin synthesis. Cyclooxygenase exists in two isoforms (26). COX-1 is a house-keeping gene constitutively expressed in most human cells. COX-2 is a highly inducible cyclooxygenase isoform. Various proinflammatory stimuli such as IL-1β, tumor necrosis factor-α, and interferon-γ have been reported to increase COX-2 expression in many biological systems including bronchial cells (27–29). The COX-2 promoter contains a PPRE, thus PPAR-γ agonists including anti-inflammatory drugs may influence COX-2 transcription and expression. 15Δ12,14-Prostaglandin J2, thiazolidinediones, and non-steroidal anti-inflammatory drugs have been reported to alter COX-2 expression (22, 24, 30, 31). As mentioned cPLA2 activation might increase expression of genes containing PPRE in promoter regions. This hypothesis has been proved using an artificial reporter gene as described elsewhere (4). The purpose of this study was to investigate whether Group IVA cPLA2 and other phospholipases (such as secreted phospholipase A2, Group IIA, V, or X) might influence IL-8 and COX-2 gene and protein expression in human lung cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**A549 cells, a human adenocarcinoma cell line, were obtained from ATCC (American Type Culture Collection, Manassas, VA) and were grown in Ham’s F-12K medium (BIOSOURCE) with 10% fetal bovine serum (BIOSOURCE). All experiments were performed when cells were 80–90% confluent.

Methyl arachidonoyl phospholipase (MAFP) was obtained from Calbiochem. Bromoelanol lactone (BEL), an iPLA2 inhibitor, and thioetheramide-PC, an sPLA2 inhibitor, were purchased from Cayman Chemicals (Ann Arbor, MI).

**Transient Transfection Assay—**The cPLA2 overexpression plasmid was obtained from Dr. J. D. Clark and J. L. Knopf at the Genetics Institute, Boston, MA (22). The sPLA2 Group IIA expression vector in pcDNA3.1 (Invitrogen) was obtained as described previously (4). The pcDNA3.1 (Invitrogen) was obtained as described previously (4). The sPLA2 Group V and Group X vectors were obtained from Dr. D. A. Bass (Institute, Boston, MA (22). The sPLA2 Group IIA expression vector in pcDNA3.1 (Invitrogen) for insertion into Ham’s F-12K medium containing 10% fetal bovine serum and 1% penicillin-streptomycin was added to control cultures. Cells were harvested with trypsin (E-PET, Gibco) for 4 h.

**Immunoblotting—**A549 cells were grown on 6-well dishes and treated with 1 μM calcium ionophore A23187 (Calbiochem) for 8 h. In experiments involving cPLA2, or sPLA2 activators as described below, cells were washed three times in ice-cold phosphate-buffered saline and lysed using Passive Lysis Buffer (Promega, Madison, WI). Cell lysate was frozen at −80 °C. Luciferase activity was measured using a luciferase assay system (Promega) with a Turner TD20 luminometer (Promega). β-Galactosidase was measured using a β-Gal enzyme-linked immunosorbent assay kit (Roche Applied Science).

**Gene Expression Measurements Using a Real Time Polymerase Chain Reaction—**Cells were grown as described above and exposed to EGF, A23187, or MAFP. Total RNA was isolated using a RNeasy kit (Qiagen, Valencia, CA). IL-8 and COX-2 expression was measured using a real timePCR mRNA quantification assay using a TaqMan system from Applied Biosystems, Foster City, CA). COX-2 probe and primer sets were obtained from Synthegen (Houston, TX). To measure COX-2 mRNA expression, the following primer sequences were used based on mRNA sequence (GenBank accession number U04636): forward primer, 5'-GCTCAAATGATGGTTGGCTAC-3'; reverse primer, 5'-GCTGGCCGCTCGTCTATGA-3'. probe sequence, 5'-TGCCAGACCCTACCGCATCGTCTCAG-3' and commercial probe (Amersham Biosciences) were used to measure IL-8 and Rnase P1 expression. 1 μg of total RNA was reverse transcribed using a reverse transcription kit from Applied Biosystems. Real time polymerase chain reaction was conducted using an Applied Biosystems kit and run on a 7900HT instrument (Applied Biosystems) according to the manufacturer's manual using RNAse P1 gene as a standard. Relative gene expression is expressed as the ratio of the gene of interest to the S2E gene. The IL-8 fold change was compared with control S2E.

**Transfection of A549 Cells with Small Inhibitory RNA (siRNA) Directed against PPAR-γ—**siRNAs targeting bases 4–23 of the PPAR-γ coding sequence (GenBank accession number NM_000277) were designed. Untemplated TSS were added to the 3'-end of each strand. The siRNAs sequences were 5'-GUUUCGUUAAUGCCUCAUUGC-3' (PPAR-γ-1) and 5'-GGGAGAUUCTT-3' (PPAR-γ-2). The single-stranded siRNAs were annealed by incubating a 100 nM concentration of each single strand in annealing buffer (100 mM potassium acetate, 30 mM Hepes, pH 7.4, 2 mM magnesium acetate) for 2 min at 90 °C and slowly cooled down to room temperature. Cells grown in 6-well plates were transfected with 100 nM siRNA duplexes using LipofectAMINE reagent (Invitrogen) (5 ml in 1 ml of culture medium) for 5 h. After transfection, medium was changed, and cells were maintained in medium with fetal bovine serum for 16 h. The effect of siRNA on PPAR-γ and -2 protein expression was assessed by immunoblotting as described above. Cells were then treated with or without A23187 or EGF as specified below. The effect of treatment of cells with siRNA duplexes on IL-8 protein levels was determined by enzyme-linked immunosorbent assay of cellular supernatants and for COX-2 protein expression by immunoblotting of cell lysates.

**Transfection of A549 Cells with siRNA Directed against cPLA2—**RNA-DNA chimeras were synthesized by Integrated DNA Technologies. The sequence used to generate 100 n mole of siRNA duplex was: 5'-AAC UCU AGG GAC GAC AAU AUU T-3' (complementary sequence), 5'-AAU GUU GCU GUC CCA AGU TT-3'. This corresponds to bases 299–319 of the cPLA2 coding sequence (GenBank accession number M68574). The annealing procedure was performed as described above. Medium (Ham's F-12 with 10% glutamine) was incubated with LipofectAMINE (5 μM) (Invitrogen) with or without siRNA duplex (final concentration, 20 nM) for 20 min at room temperature. Cells were then treated for 5 h at 37 °C. Medium was then changed to Ham's F-12 with 10% glutamine and fetal bovine serum, and the cells were incubated...
bated an additional 72 h. Cell lysate was collected for cPLA2 Western blots. The remaining cells were incubated for 8 h with medium alone, A23187 (10⁻⁶ M), or EGF (20 ng/ml). Medium was collected for IL-8 assay by enzyme-linked immunosorbent assay, and cell lysate was collected for COX-2 and cPLA2 Western blots.

Electrophoretic Mobility Shift Assay—PPRE probes were synthesized by Keystone Laboratories (Camarillo, CA) corresponding to PPRE sequences (underlined) present in the COX-2 (5'-GAGGCGACAGGTCAT-AACCCTACT-3') and IL-8 promoters (5'-GGGTCCTCAGAGGTCAGACTTGGT-3'). The inverted PPRE sequence in the COX-2 promoter is at 3599 to 3573 relative to the transcription start site and is present as bases of 3542 to 3565 in GenBank™ accession number AF044206. The IL-8 PPRE sequence represents bases −1070 to −1045 relative to the transcription start site and is present as bases 412–437 of GenBank™ accession number M28130. Single-stranded nucleotides were reannealed by heating to 95 °C for 5 min and cooled down slowly to room temperature. A549 cells were incubated with and without A23187 (10⁻⁶ M) or EGF (10 ng/ml) for 30 min, 1 h, and 2 h prior to harvest at the 2-h time point. Nuclear extracts were prepared using a nuclear extraction kit according to the manufacturer’s directions (Sigma). DNA binding was performed by incubating 3 μg of nuclear protein in a total volume of 10 μl of binding buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM MgCl₂, 0.05 μg/μl poly(dI-dC)/poly(dI-dC), 15% glycerol) and 10,000 cpm [³²P]labeled double-stranded PPRE probes for 20 min at room temperature. The specificity of protein binding to labeled probe was assessed by competition with unlabeled probe or with PPRE consensus sequence. For the competition experiments, a 100-fold excess of unlabeled probe or PPRE consensus sequence (CAAAACTAGGTCAAAGGTCA) (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the electrophoretic mobility shift assay mixture. Nuclear protein derived from cells exposed to A23187 (10⁻⁶ M) for 60 min was utilized for these experiments. Protein-DNA complexes were resolved on a 6% DNA retardation gel (Invitrogen) in 0.5× Tris-borate-EDTA buffer at 200 V for 30 min. The dried gel was exposed to x-ray film (Kodak) with an intensifying screen at −70 °C overnight or until adequate signal was developed. An Amersham Biosciences 301 computing densitometer was used to digitize images.

Statistical Analysis—Statistical analysis was performed using Microsoft Excel 2000 (Redmond, WA) software running on an iMAC computer (Apple, Cupertino, CA). Comparisons were performed using two-tailed unpaired Student’s t tests. Values of p < 0.05 were considered statistically significant.
RESULTS

The Influence of cPLA2 Activation on IL-8 and COX-2 mRNA
Levels—Real time polymerase chain reaction was used to study
change in steady state levels of IL-8 and COX-2 mRNA after
treatment. Calcium ionophore A23187 and EGF increased both
IL-8 and COX-2 mRNA levels as shown in Fig. 1, A and B.
Treatment with the calcium ionophore A23187 resulted in an
increase in the IL-8 transcript was present even after 24 h. By this time,
the COX-2 mRNA levels had returned to control levels (Fig. 1
A and B). EGF treatment of A549 cells also resulted in an increase in
steady state mRNA levels for both IL-8 and COX-2. This effect
was transient for COX-2 and disappeared after 2 h. IL-8 mRNA
levels were still increased at the 24-h time point (Fig. 1A).

EGF treatment of A549 cells also resulted in an increase in
steady state mRNA levels for both IL-8 and COX-2. This effect
was transient for COX-2 and disappeared after 2 h. IL-8 mRNA
levels were still increased at the 24-h time point (Fig. 1A).

The Influence of cPLA2 Activation on IL-8 Reporter Gene Activity: the Effect of MAFP, a cPLA2 Inhibitor—To test
whether cPLA2 overexpression might induce IL-8 transcrip-
tion, a reporter gene assay was used. Cells were transfected
with an IL-8 reporter gene and cotransfected with a cPLA2
expression vector or empty vector. Twenty-four hours after
transfection, cells were exposed for 4 h to culture medium with
Me2SO, A23187 (10^{-6} M), or EGF (20 ng/ml) to activate cPLA2.

The Influence of cPLA2 Activation on COX-2 Reporter Gene Activity: the Effect of MAFP, a cPLA2 Inhibitor—Similar exper-
ments were performed to test the hypothesis that cPLA2 overexpression might induce COX-2 transcription. cPLA2 overexpression induced an increase of COX-2 reporter gene activity as shown on Fig. 2C. When transfected cells were stimulated with A23187 (10^-6 M) for 4 h, COX-2 reporter gene activity was increased compared with cells treated with MeSO (A23187 vehicle). These data suggest that both cPLA2 activation and cPLA2 overexpression and activation induce COX-2 reporter gene activity. Furthermore, treatment of cells with EGF produced a similar effect on COX-2 reporter gene activity. The effect of EGF was also in part inhibited by preincubation with a specific cPLA2 inhibitor, MAFP (10 μM), as shown on Fig. 2D.

The Influence of sPLA2 Group IIA, sPLA2 Group V, and sPLA2 Group X Activation on IL-8 and COX-2 Reporter Gene Activity—cPLA2 is not the only phospholipase A2 expressed in human lung cells. Therefore, we used vectors overexpressing sPLA2 Group IIA, Group V, and Group X proteins and tested whether overexpression of these enzymes might influence IL-8 and COX-2 reporter gene activity. IL-1β is known to induce the release of sPLA2 from the cells to the medium. This process is associated with an increase in sPLA2 enzyme activity in the extracellular space, activated by the calcium levels present in the medium. Previously we have shown that secretion of cells with an sPLA2 Group IIA expression vector induces an increase in arachidonic acid release (4). In Fig. 3, A and B, we present evidence that secretion of cells with sPLA2 Group V or Group X vectors, respectively, results in increased arachidonate release compared with cells transfected with empty vectors. These data suggest that these vectors produce functionally active proteins enhancing arachidonate release. Fig. 4, A, B, and C, demonstrate the lack of activation of IL-8 reporter gene in cells transfected with expression vectors encoding sPLA2 Group IIA, Group V, or Group X. In all three cases, an increase in sPLA2 activity was associated with a decrease in IL-8 reporter gene activity suggesting the possibility of an inhibitory effect of secreted phospholipase products on IL-8 transcription. Fig. 5, A, B, and C, demonstrate the influence of sPLA2 activation on COX-2 reporter gene activity. Transfection of cells with expression vectors encoding for Group IIA, V, or X isoforms of secreted phospholipases A2 did not activate COX-2 reporter gene expression. Activation of sPLA2 group A activity seems to be associated with a decrease in COX-2 reporter gene activity suggesting the possibility of an inhibitory effect of secreted phospholipase products on transcription of this gene.

Effect of Inhibitors of sPLA2, iPLA2, or cPLA2 on Spontaneous IL-8 and COX-2 Reporter Gene Activity in A549 Cells—To confirm the role of cPLA2 in the regulation of IL-8 and COX-2 expression, cells transfected with IL-8 or COX-2 reporter genes were exposed for 12 h to sPLA2, iPLA2, or cPLA2 inhibitors. Neither the sPLA2 inhibitor thioetheramide-PC nor the iPLA2 inhibitor BEL influenced IL-8 or COX-2 reporter gene activity suggesting no role for these enzymes in the regulation of IL-8 and COX-2 transcription de novo. Similarly, MAFP, a cPLA2 inhibitor, decreased both IL-8 and COX-2 reporter gene activity suggesting that cPLA2 might regulate even basal IL-8 and COX-2 transcriptional activity (Fig. 6, A and B).

Effect of Inhibitors of sPLA2, iPLA2, or cPLA2 on Calcium Ionophore and EGF-stimulated IL-8 and COX-2 Reporter Gene Activity in A549 Cells—To establish the role of cPLA2 activation by A23187 and EGF in the regulation of IL-8 and COX-2 de novo transcription, cells transfected with IL-8 or COX-2 reporter genes were preincubated for 2 h with sPLA2, iPLA2, or cPLA2 inhibitors. Afterward some cultures were exposed to A23187 (1 μM) or EGF (20 ng/ml) for 4 h (with inhibitors present in the medium) or exposed to MeSO, an A23187 vehicle, or medium, respectively. Neither the sPLA2 inhibitor thioetheramide-PC nor the iPLA2 inhibitor BEL abolished the A23187 or EGF effect on IL-8 or COX-2 reporter gene activity suggesting no role for these enzymes in the regulation of IL-8 and COX-2 transcription de novo upon calcium-dependent or EGF cell activation. MAFP, a cPLA2 inhibitor, decreased calcium ionophore-stimulated IL-8 and COX-2 reporter gene activity suggesting that cPLA2 might regulate IL-8 and COX-2 transcriptional activity (Fig. 6, C and D, respectively). Similarly, MAFP decreased EGF-stimulated IL-8 and COX-2 reporter gene activity again suggesting that cPLA2 might regulate IL-8 and COX-2 transcriptional activity (Fig. 6, E and F).

The Influence of cPLA2 Activation on IL-8 Protein Expression in A549 Cells—The observation that cPLA2 activation increases IL-8 reporter gene activity and IL-8 mRNA levels was supported by the measurements of IL-8 protein secreted by A549 cells. Exposure of A549 cells to A23187 resulted in a time-dependent increase in secretion of IL-8 as shown in Fig. 7A. A significant increase in IL-8 in the medium was present at 4 h and beyond. This effect was in part inhibited by MAFP suggesting that this effect might be cPLA2-dependent as shown
The influence of sPLA₂ Group IIA (A), sPLA₂ Group V (B), and sPLA₂ Group X (C) activation on IL-8 reporter gene activity. The influence of the aforementioned sPLA₂s upon activation with IL-1β was studied on IL-8 reporter gene activity. Subconfluent A549 cells were transfected with 1 μg of IL-8 reporter gene and 1 μg of the respective PLA₂ expression vector or an empty vector (VC), respectively. All cells were also cotransfected with 0.2 μg of β-galactosidase/CMV vector to normalize for transfection efficiency. Some cultures were incubated with IL-1β (10 ng/ml) for 4 h. Cells incubated with medium for 4 h were used as a control. Cell lysates were prepared and assayed for luciferase and β-galactosidase activity as described under “Experimental Procedures.” Data shown are mean ± S.E. (n = 4–6). *, p < 0.05 as compared with cells transfected with an empty vector.

The influence of sPLA₂ Group IIA (A), sPLA₂ Group V (B), and sPLA₂ Group X (C) activation on COX-2 reporter gene activity. The influence of the aforementioned sPLA₂s upon activation with IL-1β was studied on COX-2 reporter gene activity. Subconfluent A549 cells were transfected with 1 μg of COX-2 reporter gene and 1 μg of the respective PLA₂ expression vector or an empty vector (VC), respectively. All cells were also cotransfected with 0.2 μg of β-galactosidase/CMV vector to normalize for transfection efficiency. Some cultures were incubated with IL-1β (10 ng/ml) for 4 h. Cells incubated with medium for 4 h were used as a control. Cell lysates were prepared and assayed for luciferase and β-galactosidase activity as described under “Experimental Procedures.” Data shown are mean ± S.E. (n = 4–6). *, p < 0.05 as compared with cells transfected with an empty vector.
in Fig. 7B. A similar effect was obtained when A549 cells were exposed to EGF (20 ng/ml) as shown in Fig. 7C. The EGF effect was present at the 2-h time point. The effect of EGF was in part inhibited by MAFP as shown in Fig. 7D.

The Influence of cPLA₂ Activation on COX-2 Protein Expression in A549 Cells—The observation that cPLA₂ activation increases COX-2 reporter gene activity and COX-2 mRNA levels was supported by the assessment of COX-2 protein levels in...
A549 cells by performing immunoblotting. Treatment of A549 cells with A23187 resulted in a time-dependent increase in cellular COX-2 protein levels as shown in Fig. 8A, left panel. This effect was in part inhibited by MAFP suggesting that this effect might be cPLA2-dependent as shown in Fig. 8A, right panel. When A549 cells were treated with EGF, increases in cellular levels of COX-2 protein were also noted (Fig. 8B, left panel). This increase in COX-2 protein levels in response to EGF treatment was, in part, inhibited by pretreatment of the cells with MAFP (Fig. 8B, right panel).

**Fig. 7.** The effect of cPLA2 activation on IL-8 protein production by A549 cells. A, subconfluent A549 cells were treated with A23187 (10 μM) or Me2SO (vehicle) for the specified times. B, A549 cells were preincubated with MAFP (10 μM) for 2 h and incubated with A23187 (10 μM) for 8 h. Culture supernatants were collected, and IL-8 concentrations were measured by an immunoassay as described under “Experimental Procedures.” Data are expressed as mean ± S.E. (n = 3–6). *, p < 0.05 as compared with control. ***, p < 0.05 as compared with cells exposed to A23187 or EGF, respectively.

The effect of cPLA2 activation on IL-8 and COX-2 protein expression is decreased by siRNAs for PPAR-γ1 and PPAR-γ2. To confirm that the effect of cPLA2 activation on IL-8 and COX-2 expression is indeed via PPRE activation, we used cells transiently transfected with siRNAs for both PPAR-γ genes. Fig. 9A presents immunoblots for PPAR-γ revealing expression of both isoforms in control cells and a decrease in PPAR-γ1 and PPAR-γ2 expression in cells transiently transfected with siRNAs. These results suggest that the use of siRNA might be a useful tool in limiting PPAR-γ1 and PPAR-γ2 expression. These cells were used to study the influence of cPLA2 activation on IL-8 and COX-2 protein expression after stimulation with

**Fig. 8.** The effect of cPLA2 activation on COX-2 protein production by A549 cells. Subconfluent A549 cells were treated with A23187 for the specified times (10 μM) (A) or with EGF (20 ng/ml) (B). Some cultures were preincubated and coincubated with MAFP (right panels). Cell lysates were prepared as described under “Experimental Procedures.” 20 μg of cellular lysate was subjected to immunoblotting. Each immunoblot shown is representative of three separate experiments, each with similar results.
COX-2 protein production measured by immunoblotting. Cells were compared with control cells (cells treated with LipofectAMINE only). "Experimental Procedures," and 20 ng/ml), or medium for 8 h. Supernatants were collected and assayed for IL-8. Cells were lysed and processed as described under "Experimental Procedures." After 24 h some cultures were exposed to A23187 (10^{-6} M), EGF (20 ng/ml), or medium for 8 h. Cells were lysed and processed as described under "Experimental Procedures," and 20 μg of cell lysate was subjected to immunoblotting. A represents an immunoblot developed with anti-cPLA2 antibody. The immunoblot shown is representative of three with similar results. After 24 h some cultures were exposed to A23187 (10^{-6} M), EGF (20 ng/ml), or medium for 8 h. Cells were lysed and processed as described under "Experimental Procedures," and 20 μg of cell lysate was subjected to immunoblotting. B shows COX-2 protein production measured by immunoblotting. Cells were lysed and processed as described under "Experimental Procedures." Blots shown are representative of three with similar results.

FIG. 10. The effect of A23187 and EGF on COX-2 expression is mediated through cPLA2. Subconfluent A549 cells were transfected with siRNA against cPLA2 as described under “Experimental Procedures.” After 72 h cells were collected, and 10 μg of cell lysate was immunoblotted as described under “Experimental Procedures.” A represents an immunoblot developed with anti-cPLA2 antibody. The immunoblot shown is representative of three with similar results. After 24 h some cultures were exposed to A23187 (10^{-6} M), EGF (20 ng/ml), or medium for 8 h. Cells were lysed and processed as described under “Experimental Procedures,” and 20 μg of cell lysate was subjected to immunoblotting. B shows COX-2 protein production measured by immunoblotting. Cells were lysed and processed as described under "Experimental Procedures." Blots shown are representative of three with similar results.

A23187 or EGF. As shown in Fig. 9, B and C, transfection of A549 cells with siRNAs directed against PPAR-γ1 and PPAR-γ2 resulted in a diminished response to A23187 or to EGF in IL-8 and COX-2 protein expression suggesting that this effect is at least in part mediated through PPAR-γ1, PPAR-γ2, or both.

The Effect of Calcium Ionophore and EGF on IL-8 and COX-2 Protein Expression Is Induced by cPLA2 To confirm that cPLA2 activation may influence IL-8 and COX-2 expression we utilized cells transiently transfected with siRNAs for the cPLA2 gene. Figs. 10A and 11A present immunoblots for cPLA2 revealing its expression in control cells and a decrease in cPLA2 expression in cells transiently transfected with siRNAs. These results suggest that the use of siRNA might be a useful tool in limiting cPLA2 expression. These cells were used to study the influence of A23187 or EGF on IL-8 and COX-2 protein expression. As shown in Figs. 10B and 11B, transfection of A549 cells with siRNAs directed against cPLA2 resulted in a diminished response to A23187 or to EGF in COX-2 and IL-8 protein expression suggesting that this effect is at least in part mediated through cPLA2.

cPLA2 Activation Results in an Increase in PPAR Binding to PPRE Sequences Derived from IL-8 and COX-2 Promoters—To confirm that as a result of cPLA2 activation arachidonate or its metabolites might bind to PPAR and activate PPAR response elements, an electrophoretic mobility shift assay was performed. Probes representing PPAR response elements derived from the IL-8 or COX-2 promoters were used. Nuclear proteins were derived from cells treated with A23187 or EGF. Exposure of A549 cells to EGF increased nuclear protein binding to the probe derived from the IL-8 promoter with a maximum effect at 60 min (Fig. 12A, upper panel). Similarly nuclear protein isolated from cells exposed to A23187 exhibited an increased binding to the probe. Maximum binding was also obtained when cells were exposed to A23187 for 60 min (Fig. 12A, lower panel). Exposure of A549 cells to EGF increased nuclear protein binding to the probe derived from the COX-2 promoter with maximum effect at 60 min (Fig. 12B, upper panel). Similarly nuclear protein isolated from cells exposed to A23187 exhibited an increased binding to the probe. Maximum binding was obtained when cells were exposed to A23187 for 60 min (Fig. 12B, lower panel). These data suggest that activation of transcription of IL-8 and COX-2 may be related to an increased binding of activated PPARs to PPREs in the promoter of these genes. To confirm the specificity of the binding to the PPRE in the IL-8 and COX-2 promoters, an inhibition experiment was performed showing that a 100× excess of unlabeled probe or unlabeled PPRE consensus oligonucleotide significantly decreased the binding (Fig. 12, C and D). Both unlabeled oligonucleotide and PPRE consensus oligonucleotide markedly de-
Here we report for the first time that cPLA₂ activation induces COX-2 and IL-8 expression. Five lines of evidence support this conclusion. First, treatment of A549 cells with activators of cPLA₂, A23187 or EGF, increased activity of both COX-2 and IL-8 reporter genes. Furthermore overexpression of cPLA₂ and activation of cPLA₂ increased transcriptional activity of both COX-2 and IL-8 reporter genes. These effects were inhibited by coincubation with a cPLA₂ inhibitor, MAPF. Second, in cells transfected with the IL-8 reporter gene or the COX-2 reporter gene, coincubation the cPLA₂ inhibitor MAPF inhibited reporter gene expression. This effect was not noted with coincubation with the iPLA₂ inhibitor bromoelactone lactone or with the sPLA₂ inhibitor theoetheramide-PC. Third, treatment of cells with A23187 or with EGF increased steady state levels of IL-8 mRNA and COX-2 mRNA. These effects were inhibited by coincubation with MAPF. Fourth, incubation of A549 cells with A23187 or with EGF increased release of IL-8 protein and increased cellular COX-2 protein levels. These effects were in part inhibited by treatment with MAPF. Finally the linkage of cPLA₂ activation and IL-8 or COX-2 expression to PPARγ activation was studied in three ways. First, electrophoretic mobility shift assays using PPRE sequence from the IL-8 and the COX-2 promoters demonstrated increased shift in both sequences in response to treatment of cells with A23187 or EGF suggesting that these sequences do bind PPARs after stimulation. Second, A549 cells were treated with siRNAs directed at PPAR-γ1 and -γ2. This treatment reduced the protein levels of PPAR-γ1 and -γ2. This treatment also reduced production of IL-8 protein and COX-2 protein in response to A23187 or to EGF compared with cells treated with transfecting reagent alone. Third, we used cells with diminished cPLA₂ expression (using siRNA) to confirm that the effect of A23187 and EGF on IL-8 and COX-2 protein expression is at least in part mediated through cPLA₂ activation. This again suggests that cytosolic phospholipase A₂ might play a role in regulation of IL-8 and COX-2 expression.

Interestingly there are no data available so far suggesting a role of other phospholipases A₂ such as Groups IIA, V, and X or iPLA₂ in this process. Previously we have shown that sPLA₂ Group IIA does not appear to be involved in activation gene transcription driven through PPARs (4). In this report we suggest that sPLA₂ types IIA, V, and X are not involved in activation of COX-2 and IL-8 transcription in response to A23187 or EGF. Two lines of evidence support this hypothesis. First, cotransfection of IL-8 or COX-2 reporter gene together with sPLA₂ Group IIA, V, or X did not increase reporter gene activity even after stimulation with IL-1β. Second, only MAPF, a cPLA₂ inhibitor, decreased reporter gene activity when transfected cells were exposed to various PLA₂ inhibitors in both quiescent and A23187- or EGF-stimulated cells. All of these data taken together suggest that IL-8 and COX-2 transcription is not influenced by sPLA₂ Group IIA, V, or X activation. One of the functional differences between cPLA₂ and sPLA₂ is that upon activation cPLA₂ is translocated from the cytoplasm to various cell membranes (including the nuclear envelope but excluding the outer cell membrane), whereas sPLA₂ is actively secreted into intercellular space where it is activated. These data taken together with our previous observations might suggest that the translocation allows cPLA₂ to deliver arachidonic and its metabolites to the nucleus and therefore to regulate gene expression.

Studies involving cPLA₂ α null mice have provided convincing evidence of the role of cPLA₂ in the inflammatory process in several diseases including but not limited to airway inflammation, arthritis, and acute lung injury (41–43). So far, its role in
these events was presumed to be related to prostaglandin and leukotriene synthesis. In this report, we suggest that the role of cPLA$_2$ in inflammation also might be related to a direct effect of its products on gene expression at least in part through PPAR-γ1 and -γ2 pathways.

Both stimuli (EGF and A23187) may alter gene expression...
through other pathways. EGF acting through the EGF receptor is able to activate the mitogen-activated protein kinase cascade leading to transcription factor phosphorylation. Similarly A23187 increases intracellular calcium levels, which may activate various pathways including protein kinase A. These pathways might contribute to regulation of IL-8 and COX-2 expression in a cPLA2-independent way (33–35, 44–48). Utilization of cells with decreased cPLA2 expression provided the evidence that cPLA2 or downstream products of this pathway might play a role in EGF- and calcium-dependent regulation of IL-8 and COX-2 expression in human lung cells.

Recently the role of sPLA2 Group IIA in regulating sPLA2 expression has been linked to cPLA2 activation by secreted sPLA2 in rat mesangial cells. This autocrine loop might utilize PPAR-α (36). As we have previously shown A549 cells do not express PPAR-α (4). This may explain why sPLA2 overexpression did not increase IL-8 or cyclooxygenase-2 expression in A549 cells. Further studies investigating the role of enzymes such as COX-1, COX-2, 12-lipoxygenase, and 15-lipoxygenase and other lipid products of cPLA2 activation are needed to clarify the role of cPLA2 in the regulation of gene expression.

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Cytosolic Phospholipase A$_2$ Group IV$\alpha$ but Not Secreted Phospholipase A$_2$ Group IIA, V, or X Induces Interleukin-8 and Cyclooxygenase-2 Gene and Protein Expression through Peroxisome Proliferator-activated Receptors $\gamma$ 1 and 2 in Human Lung Cells

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