85-kDa Cytosolic Phospholipidase A₂ Mediates Peroxisome
Proliﬁer-activated Receptor γ Activation in Human Lung
Epithelial Cells*

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The 85-kDa cytosolic phospholipase A₂ (cPLA₂) plays an important role in the control of arachidonic acid metabolism. This study was designed to investigate the possible contributions of cPLA₂ and group IIA secretory phospholipase A₂ (sPLA₂) in the regulation of peroxisome proliferator-activated receptor (PPAR) mediated gene transcription in human airway epithelial cells. Primary normal human bronchial epithelial cells and human lung epithelial cell lines BEAS 2B, A549, and NCI-H292 all express PPARγ and -β. Overexpression of cPLA₂ in BEAS 2B cells and primary bronchial epithelial cells resulted in a significant increase of PPARγ-mediated reporter activity. In contrast, overexpression of group IIA sPLA₂ had no effect on PPARγ activation. The PPARγ activity in A549 cells was signiﬁcantly inhibited by the cPLA₂ inhibitor arachidonyltrifluoromethyl ketone but not by the sPLA₂ inhibitor LY31727 and the iPLA₂ inhibitor HEISS. Activation of cPLA₂ by the calcium ionophore, A23187, induced a dose-dependent increase of PPAR activity in normal human bronchial epithelial cells and in the A549 cells. Electrophoretic mobility shift assays show that the binding between PPAR isolated from A549 cells and peroxisome proliferator response element (PPRE) is enhanced by A23187 but partially blocked by the cPLA₂ inhibitors arachidonyltrifluoromethyl ketone and methyl arachidonoyl fluorophosphate. Finally, NS 398, a COX-2 inhibitor, partially blocked the A23187 effect on PPAR activity and binding to the PPRE suggesting involvement of COX-2 metabolites in PPRE activation. The above results demonstrate a novel function of cPLA₂. In the control of PPARγ activation in human lung epithelial cells.

Arachidonic acid (AA) metabolism plays an important role in the pathogenesis of inﬂammation and in the regulation of

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‡ The abbreviations used are: AA, arachidonic acid; AACOCF₃, arachidonoyltrimethylammonium; CAT, chloroform; CC/21, 327, 328, 197; EMISA, electrophoretic mobility shift assay; HSS, halothan, lactone suicide substrate; NHBE, primary normal human bronchial/ tracheal epithelial cells; PLA₂, phospholipase A₂; cPLA₂, cytosolic phospholipase A₂; iPLA₂, intracellular calcium-independent PLA₂; sPLA₂, secretory PLA₂; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator response element; Me₂SO, dimethyl sulfoxide; MAFP, methyl arachidonyl fluorophosphate; PBS, phosphate-buffered saline; ANOVA, analysis of variance; GFP, green fluorescent protein; EGFP, enhanced GFP; IL, interleukin.
EXPERIMENTAL PROCEDURES

Materials—The lung epithelial cell lines BEAS 2B, A549, and H292 were obtained from the American Type Culture Collection (Manassas, VA). Frozen vials of primary normal human bronchial epithelial cells (NHBE) and the bronchial epithelial cell growth media (BEGM) were obtained from Clonetics (San Diego, CA). LHC-8 medium was obtained from BIOSOURCE International (Rockville, MD). Ham’s F12K medium, RPMI 1640 medium, fetal bovine serum, glutamine, antibiotics, and the LipofectAMINE Plus™ reagent were purchased from Invitrogen. Chloramphenicol acetyltransferase (CAT) enzyme assay system was purchased from Promega (Madison, WI). Chemiluminescent reporter assay for β-galactosidase was purchased from Tropix (Bedford, MA). The sPLA2 inhibitor LY311727 was a generous gift from Dr. E. Mihelich at Lilly Research Laboratories (Indianapolis, IN). The cPLA2 inhibitors MAFP (1 mM) and dithiothreitol (10 mM) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-linked streptavidin and chemiluminescence detection reagents were obtained from Amersham Biosciences. Plasmid purification reagents were from Qiagen (Valencia, CA). The antibodies for human cPLA2, PPAR-α, -β, and -γ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-linked streptavidin and chemiluminescence detection reagents were obtained from Amersham Biosciences. Plasmid purification reagents were from Qiagen (Valencia, CA). The antibodies for human cPLA2, PPAR-α, -β, and -γ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture—Four types of human lung epithelial cells were utilized in this study (BEAS 2B, A549, H292, and primary normal human bronchial epithelial cultures). The antibodies for human cPLA2, PPAR-α, -β, and -γ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-linked streptavidin and chemiluminescence detection reagents were obtained from Amersham Biosciences. IL-1β was obtained from R & D Systems (Minneapolis, MN). Unless indicated otherwise, all other chemicals were from Sigma. The cPLA2 expression plasmid was kindly provided by Drs. J. Clark and J. Knopf at the Department of Medicine, University of Iowa (43). The PPRE reporter construct was kindly provided by Dr. W. Wahl, Switzerland. This construct contains the CAT coding sequence driven by a promoter consisting of two copies of the CYP4A6 PPRE (2× AGGTCAAAGGTCA) upstream of the thymidine kinase minimal promoter (15).

Cell culture—Four types of human lung epithelial cells were utilized in this study (BEAS 2B, A549, H292, and primary normal human bronchial epithelial cultures). The antibodies for human cPLA2, PPAR-α, -β, and -γ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-linked streptavidin and chemiluminescence detection reagents were obtained from Amersham Biosciences. Plasmid purification reagents were from Qiagen (Valencia, CA). The antibodies for human cPLA2, PPAR-α, -β, and -γ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmid Construction—To construct the group IIA sPLA2 expression plasmid, we first obtained full-length human group IIA sPLA2 cDNA using reverse transcription-PCR of human lung RNA. The primer pair was constructed according to the cDNA sequence (44). It amplified an 823-bp product and was composed of the following sequences: 5′, CAACCTGGAGTCTCTGAGAGGCTCC; 3′, 894GCTAATT-GCTTATACAGAGACAGC (45). The amplified full-length human group IIA sPLA2 cDNA was then cloned in sense orientation into the mammalian expression vector pcDNA3.1 (Invitrogen). The identity and orientation of this construct was confirmed by DNA sequencing.

Transient Transfection of cPLA2 and Group IIA sPLA2 Expression Plasmids—The BEAS 2B and NHBE cells were used for transfection experiments. The cells were seeded on 6-well plates coated with a thin layer of type I rat tail collagen (LHC-8 medium for BEAS 2B cells and BEGM medium for NHBE cells). Transfection was performed when the cells reached ~80% confluence. The cells were co-transfected with 1.5 μg of each of PLA2 expression plasmids (cPLA2 in pMT-2 and group IIA sPLA2 in pcDNA3.1) or vectors (pMT-2 and pcDNA3.1) and 1.5 μg of PPRE reporter plasmid expressing the chloramphenicol acetyltransferase (CAT) gene using LipofectAMINE Plus™ reagent. An internal control reporter plasmid expressing the β-galactosidase gene (pGIP lacZ) was used to normalize the transfection efficiency (0.2 μg in each transfection). After exposure to the mixture of transfection reagents and plasmids for 3 (for NHBE cells) or 4 h (for BEAS 2B cells), the cultures were maintained in medium for 24 h. The cells were then incubated with or without A23187 (10⁻⁶ M) for 2 h and washed twice with phosphate-buffered saline, and the cell lysates were prepared for Western blot analysis of cPLA2 and group IIA sPLA2 as well as for measurement of CAT reporter activity.

Experimental Design—Different time points were used throughout the study in order to investigate different signal transduction events. One hour of incubation was used to investigate PPAR binding to PPREs in the EMSA experiments. A 2-h incubation after A23187 stimulation was used for reporter gene experiments where cells transinfected with overexpression vectors were used. A 4-h incubation was used for quiescent cells transfected with PPRE reporter gene only and stimulated with A23187. After 4 h, the cell lysates were added to the wells and incubated for 30 min. Cells incubated with MeSO (vehicle) served as a control. Medium was collected and centrifuged at 1000 × g for 5 min at 4 °C, and 0.9 ml of medium from each sample was transferred to a scintillation vial containing 10 ml of Bio-Safe II scintillation fluid (Research International Products Inc., Mount Prospect, IL) and counted in a scintillation counter (Beckman Instruments, Fullerton, CA) with a 15% efficiency.

Cytosolic Phospholipase A2 and Secreted Phospholipase A2 Activity Assays—cPLA2 activity was determined as described previously (35). Data are presented as [14C]arachidonic acid release in dpm/μg of cellular protein/h ± S.E. Secreted phospholipase A2 activity was measured in cell supernatants using the same system as for cPLA2, with modifications allowing detection of sPLA2 activity. sPLA2 activity was obtained as a difference between the PLA2 activity from cells supernatant untreated with dithiothreitol and the PLA2 activity from supernatant treated with 1 mM dithiothreitol. The activity assay for sPLA2 was performed in the presence of 5 mM Ca²⁺. Data are presented as [14C]arachidonic acid release in dpm/μg of cellular protein/h ± S.E.

Immunoblotting Analysis—For immunoblotting analysis of cPLA2, group IIA sPLA2, PPAR-α, PPAR-β, and PPAR-γ, the cell lysates from human airway epithelial cells were prepared using lysis buffer containing 0.05% NP-40, 1 mM EDTA, and 0.5% non-fat milk. The cell lysates containing 10 μg of cellular protein were separated on 4–20 or 16–Tris glycine gels (NOVEX, San Diego, CA) using Tris glycine SDS running buffer. The separated proteins were then electroblotted to nitrocellulose membranes (NOVEX). Non-specific binding was blocked with 3% non-fat milk in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) at room temperature for 1 h. The membranes were then incubated with primary antibodies (1:200 dilution of mouse anti-human CYP4A3 monoclonal antibody, 1:200 dilution of rabbit anti-human group IIA sPLA2 polyclonal antisemur, and 1:500 dilutions of rabbit anti-human PPAR-α, -β, and -γ polyclonal antibodies) in PBS-T containing 3% non-fat milk. After overnight incubation at 4 °C, the membranes were washed three times with PBS-T and then incubated at room temperature for 1 h with 1:5000 dilution of the corresponding horseradish peroxidase-conjugated secondary antibodies in PBS-T containing 3% non-fat milk. Following washing three times with PBS-T, the protein bands were visualized with the ECL Western blotting detection system according to the manufacturer’s instructions.

Cytosolic Phospholipase A2 Intracellular Localization during Exposure to F12K Medium—In order to investigate intracellular localization of CYP4A3 expressing cells, the CYP4A3 expressing cells (BEAS 2B, A549, H292, and primary normal human bronchial epithelial cells) were transfected with pMT-2 and pcDNA3.1 and maintained in F12K medium for 48 h. Cells were fixed in 3.7% EM grade formaldehyde obtained from Polyscience (Nalge Nunc, Naperville, IL) until 60% confluent. Cells were transfected with full-length CYP4A3 tagged with GFP (EGFP-FV) vector, which was a generous gift from Drs. J. Evans and C. Leslie from National Jewish Medical and Research Center, Denver, CO (45). After 16 h cells were washed twice with PBS and were incubated with media containing MeSO (vehicle) or with A23187 (1 μM) for 0.5 h. Afterward, cells were fixed in 5% EM grade formaldehyde obtained from Polyscience.
(Warrington, PA) for 10 min. After 3 washes in PBS in a vertical shaker, cells were fixed using ProLong Antifade Kit from Molecular Probes (Eugene, OR). Slides were analyzed using Nikon Eclipse E800 microscope (Nikon, Japan) and Scion Image software (Scion Corp., Frederick, MD).

**Nuclear Protein Isolation from A549 Cells**—A549 cells were grown in T-150 flasks to 90% confluence. Cells were exposed to A23187 (1 μM) for the specific times. In some cases cells were pretreated with AAOCCF₃, MAFP, or NS 398 for 2 h prior to treatment with A23187. Culture medium was removed, and cells were washed 3 times with ice-cold PBS, harvested by scraping into 4 ml of PBS, and centrifuged (500 × g, 5 min). The pelleted nuclei were isolated in a high salt buffer (20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, aprotinin, pepstatin, and leupeptin, each 2 μg/ml). After 15 min on ice, Nonidet P-40 was added to a final concentration of 0.6% (v/v), and the nuclei were pelleted by centrifugation (5000 × g, 5 min). The pelleted nuclei were dispersed in a high salt buffer (20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 1 mM dithiorthiol, 1 mM phenylmethylsulfonyl fluoride, aprotinin, pepstatin, and leupeptin, each 2 mg/ml) to solubilize DNA-binding proteins. The suspended nuclei were harvested by scraping into 4 ml of PBS, and centrifuged (500 g) were utilized.

**EMSAs**—Double-stranded PPRE oligonucleotides (CATAAACCTAGTT-CAAAGGTTCA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The PPRE probes were labeled with γ-³²P]ATP (Amersham Biosciences) using T4 polynucleotide kinase (Promega) and purified on G-50 columns (Amersham Biosciences). Nuclear extracts (5 μg) were incubated with the ³²P-labeled PPRE oligonucleotide probe (0.5–1 × 10⁶ cpm) in binding buffer (10 mM HEPES, pH 7.8, 5% glycerol, 1 mM dithiorthiol, 1 mM phenylmethylsulfonyl fluoride, aprotinin, pepstatin, and leupeptin, each 2 mg/ml) to solubilize DNA-binding proteins. The suspended nuclei were gently shaken horizontally for 30 min at 4 °C and centrifuged in a microcentrifuge (12,000 × g, 20 min). The supernatants containing nuclear proteins were stored at −70 °C until used for EMSA. Protein concentrations were determined using a BCA assay kit (Fierce) with bovine serum albumin as a standard.

**RESULTS**

**Human Airway Epithelial Cells Express Both PPARγ and PPARβ but Not PPARα**—To determine the expression profile of PPARs in human airway epithelial cells, we first performed Western blot analysis for the three PPAR isoforms (PPARα, -β, and -γ) in primary cultures of human bronchial epithelial cells, and in cell lines BEAS 2B, A549, and H292. As shown in Fig. 1, although PPARα is highly expressed in human hepatocytes, it is not detected in human airway epithelial cells by Western blot analysis. In contrast, PPARβ and PPARγ are expressed in all four types of human airway epithelial cells. The primary human bronchial epithelial cells express higher levels of PPARβ than the cell lines BEAS 2B, A549, and H292, which is consistent with the hypothesis that PPARβ may play a role in the differentiation of airway epithelial cells (46). Two isoforms of PPARγ (PPARγ₁ and PPARγ₂, which are produced by alternative splicing of the same PPARγ gene) are present in all four types of human bronchial epithelial cells. Whereas primary normal human bronchial epithelial cells express higher PPARγ₂ than PPARγ₁, the three human airway epithelial cell lines express slightly higher levels of PPARγ₁ than PPARγ₂. As expression of PPARγ is detected in all the four types of human airway epithelial cells, we examined the contribution of cPLA₂ to PPARγ-mediated gene transcription by using a PPRE reporter construct containing the CAT coding sequence driven by a promoter consisting of two copies of the CYP4A6 PPRE in the upstream region of the thymidine kinase (TK) minimal promoter (15).

**Overexpression of cPLA₂ Increases PPARγ-mediated Gene Transcription in Human Airway Epithelial Cells**—BEAS 2B cells were transfected with pMT-2 expression vector containing cPLA₂-coding sequence or with empty pMT-2. Western blot analysis of cells transfected with the cPLA₂ expression vector demonstrated increased cPLA₂ protein expression as compared with cells transfected with empty vector (Fig. 3A). BEAS 2B cells transfected with the cPLA₂ expression vector demonstrated increased [³H]arachidonic acid release after stimulation with calcium ionophore suggesting increased calcium-dependent phospholipase activity as compared with cells transfected with an empty vector (Fig. 3B). In order to determine whether the increased cellular arachidonate release is due to increased expression of cPLA₂, an assay of cellular lysate for cPLA₂ activity was performed. In cells transfected with the cPLA₂ expression vector, cPLA₂ activity was significantly increased compared with cells transfected with an empty vector (Fig. 3C). In order to determine whether an increase in cPLA₂ activity was associated with an increase in PPRE binding, cells were co-transfected with the PPRE reporter gene and then were stimulated with calcium ionophore A23187 for 2 h to allow PPAR binding to PPRE and transcription and translation of CAT protein. Similarly, cells transfected with an empty vector were also co-transfected with the PPRE reporter gene. These cells were also stimulated with A23187. Based on the previously documented calcium-induced cPLA₂ translocation to nuclear envelope (7, 39–42), we predicted that cells with cPLA₂ overexpression would likely have increased AA and eicosanoid production in the nuclei for PPAR activation.
in response to the calcium-mobilizing agents. Cells transfected with the cPLA$_2$ expression vector exhibited greater PPRE reporter gene activity (Fig. 3D) as compared with cells transfected with control vector. Similar data were obtained from normal human bronchial cells (Fig. 4, A and B).

**Overexpression of Type IIA sPLA$_2$ Failed to Increase PPAR$\gamma$-mediated Gene Transcription in Human Airway Epithelial Cells**—We examined the possible effect of transient overexpression of group IIA sPLA$_2$ on PPAR$\gamma$ activation by using a similar approach. Western blot analysis of the cell lysate collected 24 h after transfection showed that the cells transfected with the group IIA sPLA$_2$ expression plasmid exhibited significantly increased levels of group IIA sPLA$_2$ protein (Fig. 5A). Overexpression of type IIA sPLA$_2$ increased arachidonate release as compared with cells transfected with an empty vector (Fig. 5B). In order to examine if this effect is mediated by group IIA sPLA$_2$, specific sPLA$_2$ activity was measured in the cellular supernatant from resting transfected cells and after stimulation with IL-1$\beta$. IL-1$\beta$ is a well known factor influencing sPLA$_2$ II A activity and releases the enzyme into the extracellular space. Cells transfected with the sPLA$_2$ expression vector produced more sPLA$_2$ activity compared with cells transfected with control vector (Fig. 5C). This effect was consistent when the cells were incubated with IL-1$\beta$. As the activity of group IIA sPLA$_2$ might be increased in the presence of calcium, the cells co-transfected with the sPLA$_2$ expression plasmid and the reporter plasmid were also stimulated with calcium ionophore A23187 for 2 h. As shown in Fig. 5D, group IIA sPLA$_2$ overexpression failed to increase PPRE reporter activity in response to calcium ionophore stimulation. Similar results were obtained when experiments were performed in the absence of calcium ionophore A23187 stimulation and in primary human bronchial epithelial cells (data not shown). As shown on Fig. 5E stimulation of lung cells with IL-1$\beta$ produced a small change in PPRE reporter gene activity. The change did not reach statistical significance. Therefore, the above experiments with overexpression of PLA$_2$ demonstrated an important role of cPLA$_2$, but not group IIA sPLA$_2$, in the production of AA metabolites.
for PPAR activation in human airway epithelial cells. Inhibitors of cPLA2, but Not Group IIA sPLA2, Block the PPAR-mediated Gene Transcription in Human Lung Epithelial Cells.

**FIG. 6.** The cPLA2 inhibitor AACOCF3 blocks the PPRE reporter activity in human lung epithelial cells. A, the effect of various PLA2 inhibitors on PPRE reporter activity. The A549 cells were transfected with the PPRE reporter plasmid and the β-galactosidase expression vector. Following transfection, the cells were cultured in the presence of different PLA2 inhibitors as indicated for 24 h. The cells were then lysed, and the cell extracts were obtained for measurement of CAT reporter activity. Although there was no significant difference in the measured CAT reporter activity between control cells and cells treated with the sPLA2 inhibitor LY311727 and iPLA2 inhibitor HELSS, the CAT reporter activity in cells treated with the cPLA2 inhibitors AACOCF3 was significantly decreased when compared with that in control cells (*, p < 0.05). The data were expressed as mean ± S.E. from four experiments. B, the dose-response effect of AACOCF3 on PPRE reporter gene activity. The A549 cells transfected with the PPRE reporter plasmid were cultured in the presence of different concentrations of the cPLA2 inhibitor AACOCF3 (10, 25, and 50 μM) for 24 h. AACOCF3 induced a dose-dependent inhibition of the PPRE reporter activity in A549 cells (**, p < 0.01). The results were obtained from four separate experiments.

**FIG. 5.** Overexpression of group IIA sPLA2 failed to increase PPRE reporter activity in human airway epithelial cells. A, immunoblot analysis for group IIA sPLA2 protein expression in BEAS 2B and A549 cells. Equal amounts of cellular proteins (10 μg) isolated from the BEAS 2B cells transfected with the group IIA sPLA2 expression vector or control vector (empty pcDNA3.1) were used for Western blot analysis. The blot shown is representative for three separate experiments with identical results. B, arachidonate release from cells transfected with the group IIA sPLA2 expression vector (closed bars) or control vector (open bars). BEAS 2B cells were transfected with the sPLA2 expression vector or control vector and labeled with [3H]AA as described under the “Experimental Procedures.” Cells were incubated with Me2SO or A23187 (10⁻⁶ M). Supernatants were collected and counted in a scintillation counter. Data expressed as dpm ± S.E.; *, p < 0.05 as compared with cells transfected with control vector, n = 4. D, PPRE reporter activity in cells with or without group IIA sPLA2 overexpression. The BEAS 2B cells were transfected with the group IIA sPLA2 expression vector or the control vector with co-transfection of the PPRE reporter plasmid. Following transfection, the cells were cultured for 24 h and stimulated with A23187 (10⁻⁶ M) for 2 h. The cell extracts were then prepared and processed to measure the CAT reporter activity as described under “Experimental Procedures.” The cells with group IIA sPLA2 overexpression failed to show increased CAT reporter activity when compared with the control cells (n = 4). E, the influence of sPLA2 on the PPRE reporter gene was assessed after IL-1β stimulation. After transfection with control vector (open bars) or with type IIA sPLA2 expression vector (closed bars) cells were incubated with IL-1β (1 ng/ml) for 24 or 4 h. Cells incubated with media served as a control. Cells were collected, and CAT and β-galactosidase activity were measured as described under “Experimental Procedures.” There were no differences between groups regarding PPRE reporter gene activity; n = 5–6.
cells, stimulation by A23187 (10^{-6} M) caused transient cPLA_2 accumulation predominantly in the perinuclear region (Fig. 7, A and B) suggesting that cPLA_2-derived metabolites might play an important role in gene transcription.

The Calcium Ionophore A23187 Increases Arachidonate Release and the PPARγ-mediated Gene Transcription in Human Lung Epithelial Cells—Because cPLA_2 is translocated from cytoplasm to the nuclear envelope in response to an intracellular calcium increase, we hypothesized that treatment of cells with ionophore A23187 would lead to nuclear translocation and activation of cPLA_2 and thus increase AA release in the nuclei for PPAR activation. To test this hypothesis, A549 cells (with a higher level of cPLA_2 protein expression) were transfected with the PPRE reporter construct and then stimulated with A23187 (10^{-8}, 10^{-7}, and 10^{-6} M) for 4 h. The cell lysates were collected, and the PPRE CAT reporter activity was measured. Fig. 8A represents arachidonate release from [3H]AA-labeled cells upon stimulation with A23187 (10^{-8}, 10^{-7}, and 10^{-6} M). This stimulation induced a dose-dependent arachidonate release from primary bronchial epithelial cells (data not shown) and A549 cells (Fig. 8A). As shown in Fig. 8, B and C, A23187 induced a dose-dependent increase of PPRE reporter activity in A549 cells and primary bronchial epithelial cells, respectively. This result further supports the role of calcium-mediated cPLA_2 activation in PPAR-mediated gene transcription in airway epithelial cells.

Incubation of A549 Cells with Arachidonic Acid Failed to Induce PPRE Reporter Gene Activity—Several reports suggested that extracellular delivery of arachidonic acid may induce PPRE-dependent gene transcription. Cells transfected with the PPRE reporter gene were incubated for 4 h with arachidonate in three concentrations of 10^{-5}, 10^{-6}, and 10^{-7} M. Although a trend to increased PPRE reporter gene activity was observed, it failed to reach significance as shown on Fig. 9. These data suggested that, at least in this experimental model and within this dose range, extracellular delivery of arachidonate might not influence transcription of PPRE-dependent genes.

PPAR in Human Lung Epithelial Cells Directly Binds to PPRE Oligonucleotide—The above results with a PPRE reporter construct demonstrated an important role of cPLA_2 in the activation of PPAR in human airway epithelial cells. We then utilized EMSA to examine the direct interaction between PPAR and PPRE. As shown in Fig. 10A, PPAR in the A549 cell nuclear protein is able to bind the PPRE probe. The binding specificity was confirmed by inhibition of binding with an excess of unlabeled PPRE probe and no inhibition with an excess of irrelevant oligonucleotide (TFIID consensus sequence). This result presents a direct interaction between airway epithelial cell-derived PPAR and PPRE consensus sequence.

The Binding of PPAR to PPRE in Human Airway Epithelial Cells Is Increased by Ionophore A23187 but Partially Blocked by cPLA_2 Inhibitors—To test if activation of cPLA_2 by the ionophore, A23187, alters the binding between PPAR and PPRE, EMSA was performed using nuclear protein obtained from A549 cells treated with A23187 (10^{-6} M). The binding of PPAR to PPRE was enhanced after A23187 treatment, and this effect peaked after 60 min of treatment (Fig. 10B). To demonstrate further the involvement of cPLA_2-mediated AA release in the PPAR and PPRE interaction, experiments were performed using nuclear protein isolated from A549 cells treated with cPLA_2 inhibitors, AACOCF_3, or MAFP. As shown in Fig. 10C, the A23187-induced PPAR binding was reduced by incubation with the cPLA_2 inhibitor, AACOCF_3. This effect is dose-dependent as shown in Fig. 10D. Another cPLA_2 inhibitor, MAFP, had a similar effect on PPAR binding as shown in Fig.
The above results suggest a role of cPLA2 activation in the regulation of the PPAR-PPRE interaction.

NS 398, a Cyclooxygenase-2 Inhibitor, Partially Inhibits A23187-dependent PPRE Reporter Gene Activation and Partially Blocks the Binding of PPAR to PPRE in Human Airway Epithelial Cells — A cyclooxygenase product 15-deoxy-Delta(12,14)-prostaglandin J2 is thought to be one of the PPRE agonists. We tested the role of cyclooxygenase-2 products in PPRE reporter gene activation and PPRE binding. NS 398 at least partially blocks A23187-dependent PPRE activation as shown on Fig. 11A. Gel shift assay (shown on Fig. 11B) revealed that NS 398 blocks A23187-dependent PPAR binding, suggesting the involvement cyclooxygenase-2 products in this process. This suggests that cyclooxygenase products at least partially derived from cPLA2 metabolites may play a role in PPRE activation. Blocking cyclooxygenase-2 activity might influence expression of PPRE-mediated genes.

DISCUSSION

PPARs belong to the superfamily of ligand-activated nuclear transcription factors (11–14), which regulate the expression of target genes by binding to PPRE or by interacting with other intracellular signaling molecules AP-1, NF-κB, and STAT proteins (47–49). Although PPARβ and γ are expressed in the lung, the cellular expression pattern of PPARs in lung epithelial cells is not known. In this study, we examined the expression pattern of PPAR isoforms in lung epithelial cells. Although PPARα is not detected in human lung epithelial cells, both
known prior to this study. In this study, by overexpression of cPLA2 and group IIA sPLA2 as well as utilization of chemical inhibitors of PLA2s, we demonstrated that activity of cPLA2, but not the group IIA sPLA2, played an important role in the regulation of PPARγ-mediated gene transcription in human airway epithelial cells. These findings reveal a previously unrecognized function of cPLA2, the cPLA2-regulated production of eicosanoids, and the activation of PPARs in nuclei for gene transcription.

The trans-activation of PPRE-containing genes in cells is regulated by the level of PPAR protein, the presence of specific co-activator/co-repressor, and the availability of endogenous ligands. Activation of PPAR involves ligand-induced conformational change which subsequently alters the binding of PPAR with other nuclear proteins and the basal transcriptional machinery. In addition to the role of cPLA2 in PPAR activation (as demonstrated by the PPRE reporter activity assay), our results also demonstrate an important role of cPLA2 in the interaction between the PPAR and PPRE in human airway epithelial cells (as demonstrated by electrophoretic mobility shift assays). The latter finding is consistent with the observation that AA enhances the binding of PPAR to PPRE oligonucleotides in other cells including HepG2 cells (human hepatoma cell line) (58) and Caco-2 cells (human intestinal cell line) (59). Because cPLA2 is a rate-limiting key enzyme for the release of AA from membrane phospholipids, the AA-induced PPAR-PPRE interaction and PPAR activation underscores the importance of cPLA2 in PPAR-mediated gene transcription. We were unable to demonstrate an effect of AA on PPRE reporter gene activity using extracellular delivery of AA in A549 cells. These data taken together suggest the possibility that predominantly the intracellular pool of AA derived through cPLA2 is involved in PPAR activation.

The different effects of cPLA2 and group IIA sPLA2 on PPAR activation can be explained by their different enzyme characteristics. One of the most important characteristics of cPLA2 regulation is its calcium-dependent translocation from cytoplasm to membrane (preferentially nuclear envelope (7, 39–42)), which is mediated by its N-terminal Ca2+-dependent lipid binding domain (CaLB or C2 domain) (43, 60). This is in contrast with the group IIA sPLA2, which exists either as a soluble form (located in extracellular space) or a cell-associated form (3, 8, 61, 62). Although the group IIA sPLA2 requires Ca2+ for catalytic activity, it lacks the Ca2+-dependent membrane association. Therefore, the above unique enzyme characteristics of cPLA2 and group IIA sPLA2 likely explain the different regulatory roles of these two enzymes in PPAR activation. As cPLA2 protein requires Ca2+ for its nuclear translocation, calcium ionophore A23187 was used in this study for maximal enzyme activation. When experiments with cPLA2 overexpression were performed in the absence of ionophore A23187, the cPLA2-induced increase of PPRE reporter activity was less prominent. For experiments with overexpression of group IIA sPLA2, a similar degree of PPAR activation was observed in the presence or absence of ionophore A23187. We employed IL-1β stimulation of cells transfected with group IIA sPLA2 in order to achieve the its maximum release and activation as shown in Fig. 5C. Even under these conditions (increasing sPLA2 activity) PPRE reporter gene activity did not change supporting the aforementioned hypothesis. In the A549 cells (without PLA2 overexpression), ionophore A23187 increased the PPRE reporter activity (Fig. 8) and enhanced the direct binding between PPAR and PPRE (Fig. 10). These observations again highlight the importance of calcium-mediated translocation of cPLA2 in PPAR-mediated gene transcription. It is possible that the effect on PPAR activation might depend on expression and
Fig. 10. A23187 enhances PPAR binding to PPRE. This effect is blocked by cPLA₂ inhibitors AACOCF₃ and MAFP. A, PPAR from human lung epithelial cells binds specifically to PPRE oligonucleotide (Oligo). Nuclear protein isolated from A549 cells was used for EMSA. From each sample, 3 μg of nuclear protein were incubated with ³²P-labeled PPRE consensus oligonucleotide. The specificity of the binding reaction was assessed by addition of a 200-fold excess of unlabeled PPAR or and a 100-fold excess of an irrelevant (TFIID consensus sequence) oligonucleotide 20 min before addition of the labeled probe. The arrow indicates protein-DNA complexes. The autoradiograph is representative of three separate experiments. B, A549 cells were incubated with A23187 (10⁻⁶ M) for 30, 60, or 120 min. Cells incubated without A23187 served as a control. Nuclear protein was extracted for EMSA as described under “Experimental Procedures.” From each sample, 3 μg of nuclear protein were incubated with ³²P-labeled PPAR oligonucleotide probe. The autoradiograph is representative of three experiments each with similar results. The arrow indicates protein-DNA complexes. C, the effect of the cPLA₂ inhibitor, AACOCF₃, on PPAR binding. A549 cells were incubated with Me₂SO (10 μM) or preincubated (for 30 min) with AACOCF₃ (10 μM) followed by an incubation with A23187 (10⁻⁶ M) or media for 1 h. Cells were harvested, and nuclear protein was extracted for EMSA as described under “Experimental Procedures.” From each sample, 3 μg of nuclear protein were incubated with ³²P-labeled PPAR oligonucleotide probes. The arrow indicates protein-DNA complexes. The autoradiographs are representative of three separate experiments each with similar results. D, the cPLA₂ inhibitor, AACOCF₃, inhibits PPAR binding in a dose-dependent manner. A549 cells were incubated with Me₂SO or preincubated (for 30 min) with AACOCF₃ (10, 25, and 50 μM) followed by an incubation with A23187 (10⁻⁶ M) or media with Me₂SO for 1 h. Cells were harvested, and nuclear protein was extracted for EMSA as described under “Experimental Procedures.” From each sample, 3 μg of nuclear protein were incubated with ³²P-labeled PPAR oligonucleotide probes. The arrow indicates protein-DNA complexes. The autoradiographs are representative of three separate experiments each with similar results. E, the effect of the cPLA₂ inhibitor MAFP on PPAR binding. A549 cells were preincubated (for 2 h) with MAFP (10 μM) followed by an incubation with A23187 (10⁻⁶ M) or media for 1 h. Cells were harvested, and nuclear protein was extracted for EMSA as described under “Experimental Procedures.” From each sample, 3 μg of nuclear protein were incubated with ³²P-labeled PPAR oligonucleotide probes. The arrow indicates protein-DNA complexes. The autoradiographs are representative of three separate experiments each with similar results.
activity of various types of phospholipases. Enzymes which are mostly active intracellularly (like group IV) might be responsible for PPAR activation, whereas secreted PLA₂ (like group IIA and V) may not be involved in this process, although this speculation needs to be supported by experimental data. These results, along with the recent study showing the induction of PLA₂ expression by PPARα in a preadipocyte cell line (63), unveil a novel feedback control between PLA₂ and PPAR in human cells. We also demonstrated that NS 398, a COX-2 inhibitor, at least partially blocks the effect of PLA₂-mediated PPARγ activation and decreased binding to the PPRE as confirmed by a gel shift assay. It has been demonstrated that there is a functional coupling between PLA₂ and COX-2 (62, 64, 65). Our data suggest that the effect on PPAR activation might take place through COX-2 metabolites at least in this experimental model, although further studies are needed to explore this matter.

In summary, this study demonstrates an important role of PLA₂, but not group IIA sPLA₂, in the control of PPARγ activation in human bronchial epithelial cells. As PLA₂ plays an important role in mediating airway inflammation and PPARγ has been shown to possess anti-inflammatory functions, the PLA₂-mediated PPARγ activation likely represents a novel mechanism for the feedback control of airway inflammation. Furthermore, in light of the role of PPAR in the airway epithelial cell differentiation and lung cancer cell differentiation/apoptosis, the PLA₂-mediated PPAR activation may also provide a potential link between airway inflammation and other important aspects of airway epithelial cell biology such as differentiation and carcinogenesis. Further studies investigating the biological implications of arachidonic acid metabolism in PPAR activation in airway epithelial cells may provide important information on the pathogenesis of airway disorders.

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