Interferon-γ Induces the Synthesis and Activation of Cytosolic Phospholipase A_2

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Abstract

Both IFN-α/β and IFN-γ have recently been demonstrated to induce a rapid but transient activation of phospholipase A_2 (PLA_2) in BALB/c 3T3 fibroblasts and a human neuroblastoma cell line. We report that IFN-γ induces the synthesis and prolonged activation of cytosolic phospholipase A_2 ($cPLA_2$) in a human bronchial epithelial cell line (BEAS 2B). Treatment of the cells with IFN-γ (300 U/ml) increased the release of [^{3}H]arachidonic acid (AA) from prelabeled cells with a maximal effect at 12 h after stimulation. The increased [^{3}H]AA release was inhibited by the PLA_2 inhibitor *p*-bromophenacyl bromide (10^{-5} M). Calcium ionophore A23187 (10^{-5} M) further increased the [^{3}H]AA release from the IFN-γ–treated cells. Subcellular enzyme activity assay revealed that IFN-γ increased PLA_2 activity in both the cytosol and membrane fractions with a translocation of the $cPLA_2$ to cell membranes in a Ca^{2+}-free cell lysing buffer. Treatment with IFN-γ also induced the release of 15-HETE, an arachidonic acid metabolite. Immunoblot showed that IFN-γ induced the synthesis of $cPLA_2$ protein. Nuclear run-on assay demonstrated that IFN-γ initiated $cPLA_2$ gene transcription within 15 min, and this effect was sustained at 4 h and returned to near control level at 12 h. The $cPLA_2$ mRNA level was assayed by reverse transcription and PCR. IFN-γ was found to increase the $cPLA_2$ mRNA after 2–24 h treatment. Furthermore, the IFN-γ induced $cPLA_2$ mRNA increase was blocked by inhibitors of protein kinase C and calcium/calmodulin-dependent protein kinases, suggesting the involvement of these protein kinases in IFN-γ–induced gene expression of $cPLA_2$. This study shows that IFN-γ induces the synthesis and prolonged activation of $cPLA_2$. (*J. Clin. Invest.* 1994. 93:571–577.) Key words: interferon-γ • cytosolic phospholipase A_2 • arachidonic acid • epithelial cells

Introduction

IFNs, a heterogeneous family of cytokines, were initially defined by their ability to interfere with viral replication. In addition to antiviral properties, IFNs are now known to induce a variety of other physiological responses including growth inhibitory and immunoregulatory activities (1). The IFNs exert their multiple activities primarily by inducing the synthesis of proteins. IFN-α/β (type I interferon) and IFN-γ (type II interferon) have different cell surface receptors and each induces the expression of a unique set of genes in addition to a common set. IFN-α treatment of BALB/c 3T3 fibroblasts induces a rapid but transient stimulation of phospholipase A_2 (PLA_2)[1] activity (2). The PLA_2 inhibitor, *p*-bromophenacyl bromide, blocks the IFN-α–induced binding of nuclear factors to the interferon-stimulated response element of 2-5A synthetase gene. Treatment of the cells with nordihydroguaiaretic acid, a lipoxygenase inhibitor, or indomethacin, a cyclooxygenase inhibitor, leads to an increase in the binding of nuclear factors to the interferon-stimulated response element of the 2-5A synthetase gene. These results suggest that PLA_2 activation is involved in IFN-α–induced gene transcription. Recently, IFN-γ has also been found to induce a rapid but transient activation of PLA_2 in a human neuroblastoma cell line (3).

PLA_2 are distinct families of enzymes that catalyze the hydrolysis of the sn-2-ester bond of phospholipids, resulting in the production of free fatty acids and lysophospholipids. The structural and mechanistic properties of the Ca^{2+}-dependent 14-kD secreted forms of PLA_2 have been extensively studied. However, as these enzymes fail to selectively hydrolyze arachidonyl-containing phospholipids, their primary function is not to initiate the biosynthesis of prostaglandins and leukotrienes, the biologically active lipid mediators (4–5). Recently, a new group of arachidonic acid (AA)-selective cytosolic PLA_2 ($cPLA_2$) has been characterized (6–8). The $cPLA_2$ preferentially hydrolyzes phospholipids containing AA esterified in the sn-2 position, resulting in the formation of AA and lysophospholipids, the precursors of prostaglandins, leukotrienes and platelet-activating factor. The activity of $cPLA_2$ is increased in the presence of Ca^{2+} or after phosphorylation at serine, threonine, or tyrosine residues (9, 10). PDGF, EGF, ATP, thrombin, and phorbol 12-tetradecanoate 13-acetate cause phosphorylation of the serine or threonine residues of the $cPLA_2$ and result in enhanced enzyme activity (9). Phosphorylation at serine-505 by mitogen-activated protein kinase causes an increase in $cPLA_2$ activity (11, 12). Interleukin-1α and macrophage colony–stimulating factor have been recently reported to induce the accumulation of $cPLA_2$ in human fibroblasts and monocytes (13, 14). However, much remains unknown about the regulatory mechanisms of this novel enzyme.

Airway epithelium may play an active role in initiating and modulating airway inflammation (15). Freshly isolated or cultured airway epithelial cells are able to generate and release AA metabolites and PAF in response to various stimuli. Furthermore, release of these mediators may be important in the pathogenesis of disorders characterized by airway inflammation, such as asthma. In this report, the effect of IFN-γ on $cPLA_2$ was investigated in a human bronchial epithelial cell line (BEAS 2B cells).

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1. *Abbreviations used in this paper:* AA, arachidonic acid; BEAS 2B cells, human bronchial epithelial cell line; $cPLA_2$, cytosolic phospholipase A_2; HETE, hydroxyeicosatetraenoic acid; PKC, protein kinase C; PLA_2, phospholipase A_2; RT-PCR, reverse transcription and PCR; 15-LO, 15-lipoxygenase.

Methods

Cell culture. BEAS-2B cells, a human bronchial epithelial cell line transformed by an adenovirus 12-SV40 hybrid virus, was a gift from Dr. J. E. Lechner (National Cancer Institute, National Institutes of Health, Bethesda, MD) (16). The cells were maintained in serum-free, hormonally defined culture media, LHC-8 (Biofluids Inc., Rockville, MD) and grown on 175-cm^2 tissue culture flasks (Falcon Labware/Becton Dickinson, Oxnard, CA) that were coated with a thin layer of rat tail collagen type I (Collaborative Research, Bedford, MA). Experiments were performed when the cells reached confluence (~30 million cells/flask).

Arachidonic acid release. The BEAS 2B cells grown on 175-cm^2 culture flasks were labeled for 18 h with 1 μCi/ml [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (214 Ci/mmol; Amersham Corp., Arlington Heights, IL) in LHC-8 media. After repeated washing with media, 20 ml of fresh media were added to each flask. Subsequently, some cultures were exposed to IFN-γ (300 U/ml), while others were maintained as controls. After 4–24 h incubation with IFN-γ, the supernatants were harvested. The samples were extracted by Sep-Pak C_{18} cartridges and chromatographed by reverse phase HPLC, as previously described (17–19). Individual octadecyl-silane C_{18} cartridges (Sep-Pak C_{18}; Waters Associates, Milford, MA) were prepared with 15 ml of methanol followed by 5 ml of 5 mM ethylenediaminetetra-acetic acid and 10 ml of water. Samples were loaded onto the cartridges, washed with 10 ml of water, and eluted with 4 ml of methanol. The methanol fraction was collected, evaporated to dryness under steady flow nitrogen gas and resuspended in 200 μl of mobile phase A (see below) for analysis by HPLC. A column (Ultrasphere C_{18}; Beckman Instruments, Fullerton, CA) measuring 4.7 mm × 250 mm with 5-μm particle size was used. A gradient program was used with mobile phase A, water/acetonitrile/phosphoric acid (75:25:0.025); and mobile phase B, methanol/acetonitrile/trifluoroacetic acid (60:40:0.0016) at a flow rate of 1.5 ml/min. The arachidonic acid fraction of HPLC elution was collected and measured for radioactivity.

Assay of PLA_2 activity. PLA_2 activity was assayed by modification of a previously described method (20). Confluent BEAS 2B cells grown in 175-cm^2 flasks were incubated with IFN-γ. At the indicated times, treated and control cells were rinsed twice and digested with 0.1% collagenase for 10 min. After washing three times with cold HBSS(⁻), the cells were transferred to 0.5 ml of homogenization buffer: 50 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM EGTA, 50 μg/ml leupeptin, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μM phosphoramidon, 10 μg/ml soybean trypsin inhibitor, and 100 μg/ml aprotinin. Cells in the homogenization buffer were sonicated for 4 × 15 s using a microprobe. The homogenate was centrifuged at 1,000 *g* for 5 min to remove nuclei, unbroken cells, and debris. The low speed supernatant was ultracentrifuged at 100,000 *g* for 1 h to produce a soluble fraction (crude cytosol) and a particulate fraction (pellet). The particulate fraction was washed twice in homogenization buffer to remove contaminating cytosol and resuspended in homogenization buffer containing 0.05% Triton X-100. After a 5-s sonication, the particulate fraction was incubated for 30 min on ice to solubilize membrane-bound protein, and this fraction was designated as crude membrane. PLA_2 activity was assayed with sn-2 [^{14}C]arachidonyl phosphatidylcholine as substrate (53.0 mCi/mmol; New England Nuclear, Boston, MA). Substrates were dried under nitrogen and resuspended in dimethyl sulfoxide. 2 μl of substrate (final concentration = 15 μM) was pipetted into an Eppendorf microcentrifuge tube. Reactions were initiated by the addition of 2.5 μl of 116 mM calcium stock (final calcium concentration = 2.9 mM, 0.9 mM in excess of the combined EDTA and EGTA concentrations) and 95.5 μl of crude cytosol and membrane factions. The reaction mixture was incubated for 1 h at 37°C and terminated by the addition of 300 μl of 2:1 chloroform/methanol containing 1% acetic acid and 1 mg/ml free AA. Release of free fatty acid was analyzed using silica gel H thin layer chromatography plates (Analtech Inc., Newark, DE). After vigorous vortexing and a brief centrifugation, 75 μl of the chloroform phase was spotted on the plates, and the plates were developed with heptane/isopropyl ether/acetic acid (60:40:4). Under this system, free arachidonic acid is well resolved from diglycerides and unhydrolyzed phospholipids. Free AA was then scraped and quantified by scintillation counting. PLA_2 activity was calculated as picomoles of AA released per milligram of protein per hour and expressed as percent change from control. The samples were assayed for protein in triplicate (BCA Protein Assay; Pierce Chemical Co., Rockford, IL). We noted that the amount of protein was variable in particulate fractions without Triton X-100 treatment. After Triton X-100 treatment, the standard deviation of measured protein amount was greatly reduced. Therefore, we used Triton X-100 to assay the membrane-associated PLA_2 activity.

Assay of 15-hydroxyeicosatetraenoic acid (15-HETE) production. The cells grown in 175-cm^2 collagen-coated flasks were incubated with 10–1,000 U/ml of IFN-γ for indicated times. The spent media were harvested and extracted by Sep-Pak C_{18} extraction followed by HPLC separation (17–19). The elution corresponding to 15-HETE standard was collected for radioimmunoassay (15-HETE RIA kit; Amersham Corp.). Assays for intact cell 15-lipoxygenase activity were performed as described (21). The cells were treated with IFN-γ for 4–12 h and harvested after 10 min digestion with 0.1% collagenase. After washing, the cells were incubated with 10 μM [1-^{14}C]AA (55 mCi/mmol; Amersham Corp.) and 90 μM cold AA in 0.5 ml Hepes-buffered HBSS⁺ for 15–60 min. The reaction was terminated by adding 2 ml methanol. The samples were extracted by Sep-Pak C_{18} cartridges and chromatographed by HPLC to determine 15-HETE production.

Immunoblot of $cPLA_2$ protein. 20 μg of crude cytosol and membrane protein was subjected to SDS-PAGE on 8% polyacrylamide gels using Tris-glycine buffer. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane that was blocked with 5% nonfat milk overnight, then probed with a 1:1,000 dilution of rabbit anti-$cPLA_2$ (provided by the Genetics Institute, Boston, MA), and finally detected with a 1:1,000 dilution of horseradish peroxidase–labeled protein A using the electrochemiluminescence Western blotting detection system (Amersham Corp.).

Nuclear run-on assay. Nuclear run-on assays were performed using modifications of previously described methods (22, 23). Confluent, quiescent cultures of BEAS-2B cells were stimulated with IFN-γ (300 U/ml) for indicated times and digested with 0.1% collagenase in HBSS⁻ for 10 min. After washing twice with cold HBSS⁻, the cell pellet was resuspended in 4 ml NP40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (vol/vol) Nonidet P-40, 50 μg/ml leupeptin, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 100 μg/ml aprotinin), incubated for 5 min on ice, and centrifuged at 500 *g* for 5 min. The nuclear pellet was washed once with 4 ml NP-40 lysis buffer and centrifuged at 500 *g*. The supernatant was discarded, the nuclei were resuspended in 200 μl reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 1 mM dithiothreitol, 0.5 mM each of ATP, CTP, and GTP, and 200 μCi of [α-^{32}P]UTP) (3,000 Ci/mmol; New England Nuclear), and reacted at 30°C for 1 h. RNA was extracted by guanidinium thiocynate-phenol-chloroform extraction method (RNA isolation kit, Stratagene, La Jolla, CA) with the addition of 100 μg yeast carrier transfer RNA. The samples were resuspended to equal counts per minute per milliliter in hybridization buffer (3–3.5 × 10^6 cpm/ml) containing 50 mM Pipes, pH 6.8, 10 mM EDTA, 600 mM NaCl, 0.2% SDS, and 100 μg/ml denatured salmon tests DNA. Hybridization to denatured cytosolic PLA_2 and chloramphenicol acetyltransferase DNAs slot blotted on nitrocellulose filters was performed at 65°C for 40 h after prehybridization at 80°C for 2 h in hybridization buffer containing 1% SDS. After hybridization the filters were washed in 2 × SSC, 0.1% SDS for 1 h × 2, air dried, and exposed to x-ray film for 2–4 d. For binding to nitrocellulose, the PCR amplified $cPLA_2$ fragment (306 bp, see below) and chloramphenicol acetyltransferase gene DNA (Pharmacia Fine Chemicals, Piscataway, NJ) were denatured by incubation with 0.2 N NaOH for 30 min at room temperature. The DNA was spotted onto nitrocellulose using the Schleicher & Schuell, Inc. (Keene, NH) slot blot apparatus.

Reverse transcription and PCR (RT-PCR). Cells were treated with IFN-γ for 4 h with or without inhibitors. Inhibitors were added to the culture 10 min before the addition of IFN-γ (300 U/ml). Total cellular RNA was extracted from 175-cm^2 culture flasks by the single step guan-

idinium thiocyanate-phenol-chloroform extraction method. The RNA pellet was precipitated with isopropanol and washed twice with 70% ethanol, vacuum dried, and redissolved in 5 μl diethylpyrocarbonate water. Before RT-PCR, 5 μl of RNase free DNase I (10,000 U/ml; Stratagene) was added and incubated at 37°C for 30 min to digest any possible contaminating DNA. The RNase free DNase was heat inactivated at 95°C for 5 min and then cooled to 4°C. Total RNA (5 μg/reaction) was reverse transcribed into cDNA at 42°C for 15 min using Moloney murine leukemia virus reverse transcriptase and random hexamer primers (GeneAmp RNA PCR Kit; Perkin-Elmer Cetus, Norwalk, CT). The reverse transcriptase was then heat inactivated at 99°C for 5 min and the cDNA cooled to 4°C. The cDNA was amplified via PCR using *Thermus aquaticus* DNA polymerase. The reaction was carried out for 45 cycles with 1 μM of 5′ and 3′ primers in 100 μl of reaction mixture using a step program (94°C, 1 min; 55°C, 1 min; 72°C, 2 min), followed by a 10 min final extension at 72°C. The products were electrophoresed on a 1% agarose/3% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME) and stained with ethidium bromide. The cytosolic PLA_2 primer pair was constructed according to the cDNA sequence of $cPLA_2$ (7). It amplified a 306-bp PCR product and was composed of the following sequences: 5′ primer-CTCACACCACAGAAAGTTAAAAGAT (799–823); 3′ primer-AAATAAGTCGGGAGCCATAAA (1104–1084). The β-actin primer pair amplified a 510-bp product (Clontech Laboratories, Inc., Palo Alto, CA) and was composed of the following sequences: 5′ primer-GAAATCGTGCGTGACATTAAG (660–680); 3′primer-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC (1169–1137). The reverse transcription and PCR experimental conditions are identical for both $cPLA_2$ and β-actin. The PCR conditions were found to amplify cDNA molecules in a linear fashion when a series of dilution of cDNA from IFN-γ–stimulated cultures (2-, 1-, 0.5-, and 0.25-fold) were amplified using primer pairs for β-actin and $cPLA_2$ and signal quantity was determined by laser densitometry. The 306-bp PCR product of cytosolic PLA_2 was recovered from the gel and used as the probe for the nuclear run-on assay.

Materials. IFN-γ and leupeptin were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Nonradiolabeled nucleotides were purchased from Pharmacia Fine Chemicals. Staurosporine, H-7, 2-aminopurine, genistein, cycloheximide, W-7, pertusis toxin, phenylmethylsulfonyl fluoride, ionophore A23187, and nonradiolabeled AA were purchased from Calbiochem (La Jolla, CA). Aprotinin was purchased from ICN Biochemicals (Cleveland, OH). Phosphoramidon was purchased from Peninsula Laboratories (Belmont, CA). Soybean trypsin inhibitor was purchased from Biofluids Inc. (Rockville, MD). Salmon sperm DNA was purchased from Stratagene. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

The effect of IFN-γ on [^{3}H]AA release. Intact cell PLA_2 activity was measured by prelabeling the cells with [^{3}H]AA. IFN-γ induced a significantly increased release of [^{3}H]AA after 4–24 h incubation with maximal release at 12 h (Fig. 1). The IFN-γ–induced [^{3}H]AA release was inhibited by 4-bromophenacyl bromide, a PLA_2 inhibitor, but not inhibited by pertusis toxin (Fig. 2). These results demonstrate that prolonged treatment of the cells with IFN-γ activates PLA_2, which induces the release of prelabeled [^{3}H]AA.

The effect of IFN-γ on PLA_2 activity. We further measured the PLA_2 activity in cytosol and membrane fractions after IFN-γ treatment. The cells were treated with IFN-γ for 4–24 h and lysed in Ca^{2+}-free 50 mM Hepes buffer (pH 8.0) containing calcium chelators and proteinase inhibitors. At baseline culture conditions, the measured PLA_2 activities were 1,030 pmol/h per mg protein in the soluble fractions and 280 pmol/h per mg protein in the particulate fractions. IFN-γ significantly increased PLA_2 activity in both cytosol and membrane fractions

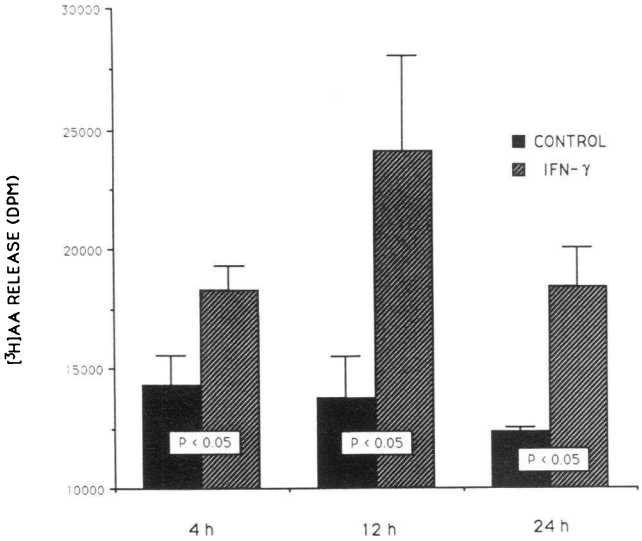

Figure 1. The release of [^{3}H]AA by BEAS 2B cells. The cells were grown on 175-cm^2 culture flasks in LHC-8 media and labeled with [^{3}H]AA (1 μCi/ml) for 18 h. After repeated washing with media, 20 ml of fresh media were added to each flask. Subsequently, some cultures were exposed to IFN-γ (300 U/ml), while others were maintained as controls. After 4-, 12-, or 24-h incubations with IFN-γ, the supernatants were harvested. The samples were chromatographed by HPLC, and AA fractions collected and measured for radioactivity. The results were expressed as the amount of [^{3}H]AA released (dpm) per flask. The values represent mean±SE of three to five separate experiments.

after a 4-h incubation. After a 12-h incubation, the membrane-bound PLA_2 activity further increased, while the PLA_2 activity in the cytosol fraction decreased toward control levels, suggesting a translocation of $cPLA_2$ to the membrane (Fig. 3).

To assess the effect of Triton X-100 on membrane-associated PLA_2 activity, experiments were also performed without Triton X-100. The calculated activities in the samples not treated with Triton X-100 were more variable than those treated with Triton X-100. Although the absolute disintegration per minute of liberated [^{14}C]AA decreased by ~30% with Triton X-100 treatment, the use of Triton X-100 gave more reproducible results when standardized for protein.

The effect of IFN-γ on 15-HETE production. 15-HETE is one of the major metabolites of AA and is produced in high amounts in human airway epithelial cells and eosinophils (21, 24). We hypothesized that increased $cPLA_2$ activity is likely to increase 15-HETE release. The cells were incubated with various concentrations of recombinant human IFN-γ (10–1,000 U/ml) for 0.5–48 h. The supernatants were extracted by Sep-Pak C_{18} cartridges and chromatographed by HPLC and quantitated by RIA. IFN-γ increased 15-HETE release at 12 and 24 h with no early effect (Fig. 4).

Additional experiments were performed to investigate the possible effect of IFN-γ on arachidonate 15-lipoxygenase (15-LO) activity in the BEAS 2B cells. The 15-LO activity in the BEAS 2B cells was low, and there was no difference in the release of [^{14}C]15-HETE between control and IFN-γ–treated cells (data not shown). These results indicated that IFN-γ had no direct effect on 15-LO activity in the intact cells. Similar results were found when 15-LO activity was assayed in the crude cytosol and membrane fractions prepared from control and IFN-γ–treated cells (data not shown).

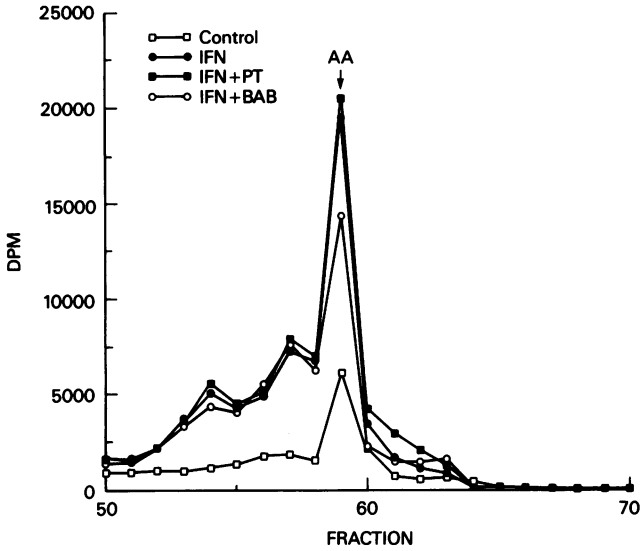

Figure 2. A representative profile of HPLC showing the effect of 4-bromaphenacyl bromide and pertussis toxin (*PT*) on IFN-γ induced [^{3}H]AA release. The cells were pretreated with 200 ng/ml pertussis toxin for 12 h and 10 μM bromophenacyl bromide (*BPB*) for 30 min before the addition of IFN-γ (300 U/ml). After a 12-h incubation, the supernatants were collected and the release of AA was determined. The retention time of AA was indicated along the top of the figure.

The effect of IFN-γ on the amount of cPLA$_2$ protein. Immunoblot of the cytosol and membrane fractions prepared from the control and IFN-γ–treated cells was performed using a specific antibody to cPLA$_2$. The cPLA$_2$ protein (110 kD) was increased in both the cytosol and the membrane fractions after treatment with IFN-γ for 4, 12, and 24 h (Fig. 5). The membrane samples contain some lower molecular weight proteins which were recognized by the anti-cPLA$_2$ antibody, suggesting that they are degradation products of cPLA$_2$ protein.

The effect of calcium ionophore A23187 on [^{3}H]AA release from IFN-γ–treated cells. As IFN-γ induces the synthesis of cPLA$_2$ protein, the exposure of IFN-γ–treated cells to a second PLA$_2$-activating stimulus, such as the calcium ionophore A23187, might result in a further increase in [^{3}H]AA release. The cells were prelabeled with [^{3}H]AA and followed by treatment with IFN-γ (300 U/ml, 12 h). After washing, the cells were incubated with or without 10^{-5} M ionophore A23187 for 15 min in HBSS containing 1.3 mM Ca^{2+}. While IFN-γ–induced [^{3}H]AA release remained increased in this 15-min incubation period, the cells stimulated with IFN-γ showed a much more remarkably increased [^{3}H]AA release in response to the subsequent ionophore A23187 stimulation (Fig. 6). This result suggests that the IFN-γ–induced cPLA$_2$ protein can be further activated by calcium ionophore A23187.

The effect of IFN-γ on cPLA$_2$ gene transcription and mRNA level. To assay whether IFN-γ increases cPLA$_2$ protein by stimulating transcription, confluent cultures of BEAS 2B cells were stimulated with IFN-γ (300 U/ml) for 15 min–12 h and the isolated nuclei analyzed by a nuclear run-on assay as described under Methods. IFN-γ stimulated the initiation of cPLA$_2$ gene transcription starting at 15 min after exposure and returning to near control level by 12 h (Fig. 7).

RT-PCR amplification of cPLA$_2$ mRNA was performed after treatment of the cells with IFN-γ and various protein kinase inhibitors. IFN-γ increased cPLA$_2$ message accumulation after 2–24-h treatment. Fig. 8 shows representative results of IFN-γ (300 U/ml, 4 h treatment) induced cPLA$_2$ mRNA increase and the inhibitory effect of various protein kinase inhibitors. The IFN-γ induced increase in cPLA$_2$ mRNA was inhibited by the broad inhibitor of protein kinases staurosporine, the protein kinase C (PKC) inhibitors sphingosine and H-7, the calcium/calmodulin-dependent protein kinase inhibitor W-7, but not by the double-strand RNA–dependent protein kinase inhibitor 2-amino purine and tyrosine kinase inhibitor genistein. The above results suggest that the IFN-γ–induced cPLA$_2$ message increase requires PKC and perhaps calcium/calmodulin-dependent protein kinases. While the IFN-γ–induced increase in cPLA$_2$ message was inhibited by actinomycin D, it was not inhibited by cycloheximide, suggesting that new protein synthesis is not required for IFN-γ–induced cPLA$_2$ transcription.

Discussion

The results presented in this paper demonstrate that IFN-γ induces the synthesis and prolonged activation of cPLA$_2$ in human bronchial epithelial cells. After treatment with IFN-γ, the cells show an increase in gene transcription, steady state mRNA levels, and protein amount of cPLA$_2$. Increased PLA$_2$ activity was demonstrated in intact cells, as well as in subcellular fractions. Treatment of the cells with IFN-γ increased the release of [^{3}H]arachidonic acid from prelabeled cells with maximal effect at 12 h after stimulation. The increased [^{3}H]AA release was inhibited by the PLA$_2$ inhibitor *p*-bromophenacyl bromide. This study demonstrates that the cPLA$_2$ is a new protein induced by the IFN family.

The cPLA$_2$ contains homology with the Ca^{2+}-dependent forms of PKC and translocates to membrane vesicles in response to physiologically relevant changes in free calcium (7, 25, 26). However, no physiological stimulus has yet been shown to induce its translocation. We further measured the PLA$_2$ activity in cytosol and membrane fractions after IFN-γ treatment. IFN-γ significantly increased PLA$_2$ activity in both cytosol and membrane fractions after a 4-h incubation. After a 12-h incubation, the membrane-bound PLA$_2$ activity further increased with a decreased PLA$_2$ activity in cytosol fraction

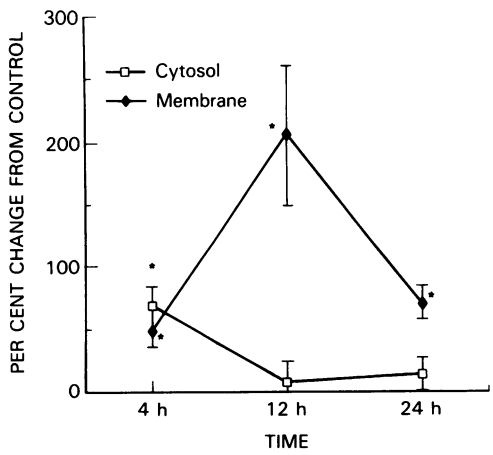

Figure 3. The effect of IFN-γ on PLA$_2$ activity. The cells grown in 175-cm^2 flasks were incubated with 300 U/ml IFN-γ. The PLA$_2$ activity in cytosol and membrane fractions was assayed as described under Methods. The results were expressed as percent increase above control. The values represent mean±SE for n = 3–11 individual experiments. *$P < 0.05$ (Student's paired t test).

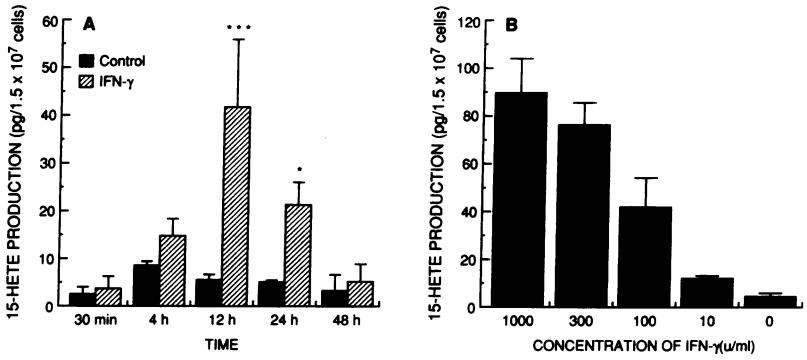

Figure 4. The effect of IFN-γ on 15-HETE release by BEAS 2B cells. The cells grown in 175-cm^2 flasks were incubated with 10–1,000 U/ml of IFN-γ for indicated times. The spent media were harvested and extracted by Sep-Pak C18 extraction followed by HPLC separation. The elution corresponding to 15-HETE standard was collected for RIA. (*A*) The time course effect of IFN-γ (100 U/ml) on 15-HETE release. IFN-γ induced a significantly increased release of 15-HETE after 12-h (***$P < 0.001$) and 24-h (*$P < 0.05$) incubations with no effects at other indicated time points. (*B*) The dose response effect of IFN-γ (12 h) on 15-HETE release. IFN-γ induced a dose-dependent increase in the release of 15-HETE ($P < 0.01$ by regression analysis). The values represent mean±SE for $n = 4$–7 individual experiments.

suggesting a translocation of $cPLA_2$ to the membrane. The mechanism of membrane association of $cPLA_2$ is not clear. As the translocation was observed in Ca^{2+} free buffer, enzyme modification such as phosphorylation might be involved.

15-HETE is one of the major metabolites of AA and is produced in high amounts in human airway epithelial cells and eosinophils (21, 24). We found that IFN-γ increased 15-HETE release at 12 and 24 h with no early effect. A recent study shows that 15-LO is induced by IL-4 in human neutrophils, and the IL-4–induced 15-LO protein is blocked by cotreatment with IFN-γ (27). We performed further experiments to investigate the possible effect of IFN-γ on 15-LO activity in the BEAS 2B cells. The results indicated that IFN-γ had no direct effect on 15-LO activity in the intact cells, as well as in the crude cytosol and membrane fractions. Thus, it appears that the effect of IFN-γ on 15-HETE release is a result of IFN-γ–induced $cPLA_2$ synthesis and activation, which provides increased amount of free AA as substrate for 15-HETE production.

As IFN-γ induces the synthesis of $cPLA_2$ protein, the exposure of IFN-γ–treated cells to a second PLA_2-activating stimulus, such as the calcium ionophore A23187, might result in a further increase in [^{3}H]AA release because: (*a*) Ca^{2+} is required for the $cPLA_2$ activity; (*b*) Ca^{2+} can promote the association of $cPLA_2$ with membrane facilitating AA hydrolysis; and (*c*) Ca^{2+} may regulate other transduction mechanisms leading to $cPLA_2$ activation. We found that the cells stimulated with IFN-γ show a markedly increased [^{3}H]AA release in response to subsequent ionophore A23187 stimulation. This may be a mechanism for the formation of airway inflammation and bronchial hyperresponsiveness, which exist in asthma, cystic fibrosis, chronic bronchitis, and atopic rhinitis (28). As IFN-γ is mainly released by T lymphocytes (29), and the T lymphocyte is one of the migratory inflammatory cells in the epithelium during airway inflammation, such as asthma (30–32), the airway epithelium may become hyperreactive by this mechanism to a variety of inflammatory mediators or external stimuli.

Signals generated by IFNs and the mechanisms by which these signals lead to gene regulation have been subjects of intense current study. While a tyrosine kinase controls IFN-α–induced gene transcription (33–35), the mechanisms of IFN-γ–induced gene transcription are not known. Since protein

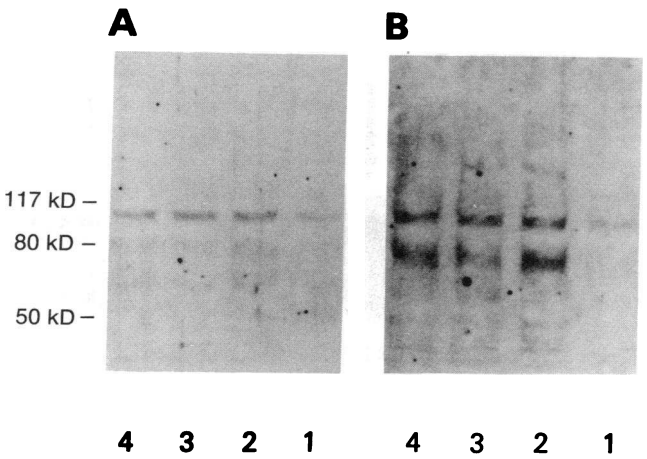

Figure 5. The effect of IFN-γ on the amount of $cPLA_2$ protein. A representative Western blot of crude cytosol (*A*) and membrane pellet (*B*) from the PLA_2 activity assay experiments. The $cPLA_2$ protein (110 kD) was shown from right as following: control cells (lane *1*), cells treated with IFN-γ (300 U/ml) for 24 h (lane *2*), 12 h (lane *3*), and 4 h (lane *4*). The size of molecular markers was indicated at the left side.

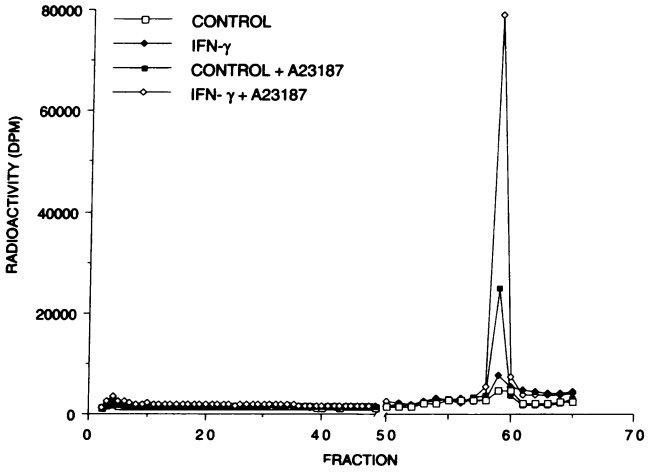

Figure 6. A representative HPLC chromatogram of ionophore A23187 induced [^{3}H]AA release from control and IFN-γ–treated cells. The cells were prelabeled with [^{3}H]AA and followed by IFN-γ treatment for 12 h. After washing the cells twice with HBSS$^+$, the cells were incubated with or without 10^{-5} M ionophore A23187 for 15 min in HBSS containing 1.3 mM Ca^{2+}. The samples were extracted by Sep-Pak C_{18} cartridges and chromatographed by HPLC. The peak shown in the figure corresponds to the retention time of AA (fraction 59).

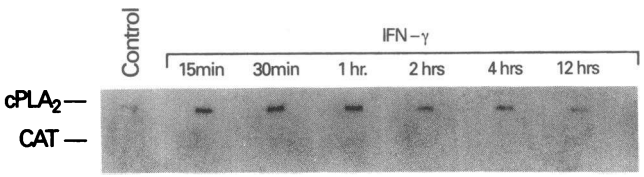

Figure 7. The effect of IFN-γ on $cPLA_2$ gene transcription. The cells were treated with 300 U/ml IFN-γ for 15 min–12 h. The nuclei were isolated and ^{32}P-labeled nuclear run-on products were hybridized to denatured $cPLA_2$ DNA probe slot-blotted on nitrocellulose filters, as described in Methods. The autoradiogram demonstrates transcriptional activation of the $cPLA_2$ gene within 15 min after the exposure of the cells to IFN-γ. This effect was sustained at 4 h and returned to near control level at 12 h. The chloramphenicol acetyltransferase (*CAT*) serves as a negative control for the specificity of hybridization conditions.

kinases are critically involved in ligand-receptor induced gene regulation, various protein kinase inhibitors were selected to investigate the effect of IFN-γ induced $cPLA_2$ gene expression. The IFN-γ–induced $cPLA_2$ mRNA increase was inhibited by staurosporine, a broad inhibitor of protein kinases, suggesting the involvement of protein kinase(s). PKC inhibitors sphingosine and H-7 blocked the IFN-γ–initiated $cPLA_2$ gene induction, suggesting the requirement of PKC. The calcium/calmodulin-dependent protein kinase inhibitor W-7 also inhibited the $cPLA_2$ expression in response to IFN-γ, suggesting the requirement of a calcium/calmodulin-dependent protein kinase. Although 2-amino purine, a double-strand RNA–dependent protein kinase inhibitor, has been found to inhibit IFN-γ–induced transcription of other genes (36), it exhibited no effect on IFN-γ–induced $cPLA_2$ gene expression. While a tyrosine kinase has been shown to directly phosphorylate the IFN-α–stimulated gene factor 3 and induce the transcription of IFN-α/β induced genes, the tyrosine kinase inhibitor, genistein, did not inhibit the IFN-γ–induced $cPLA_2$ mRNA increase. The above results suggest that the IFN-γ–induced $cPLA_2$ message increase requires PKC and calcium/calmodulin-dependent protein kinases.

The IFN-γ–induced increase in $cPLA_2$ message is not inhibited by cycloheximide, suggesting that new protein synthesis is not required for IFN-γ–induced $cPLA_2$ expression. This is consistent with the finding that IFN-γ rapidly initiates the $cPLA_2$ gene transcription (within 15 min). A number of different studies have shown that protein synthesis inhibitors potentiate and prolong the induction of early response gene expression (29, 37–39). This superinduction may be detected within 30 min or after several hours of exposure to agents such as cycloheximide or anisomycin. Cycloheximide superinduces the levels of the mRNAs by a dual effect; i.e., inhibition of the transcription shutoff by labile repressors and increased stability of the mRNAs. In this study, $cPLA_2$ gene transcription that follows a time course consistent with an early response gene is rapidly initiated. However, no superinduction of $cPLA_2$ mRNA was detected when cells were treated with IFN-γ and cycloheximide. The absence of superinduction with cycloheximide and IFN-γ may suggest that the $cPLA_2$ gene is regulated by mechanisms different from those described for the regulation of some immediate early genes. In addition, as a pertusis toxin–sensitive inhibitory guanine nucleotide binding protein Gi is involved in receptor-mediated arachidonic acid release (40), we therefore performed additional experiments to determine whether this transduction pathway is involved in the IFN-γ–induced PLA_2 activation. We found that the pertusis toxin had no effect on IFN-γ–induced [^{3}H]arachidonic acid release, suggesting that the pertusis toxin sensitive Gi is unlikely to be involved.

It has been shown that a number of agents activate $cPLA_2$ by phosphorylation of $cPLA_2$ (9, 10). Phosphorylation at serine-505 by mitogen-activating protein kinase causes an increase in $cPLA_2$ activity (11, 12). This study showed that IFN-γ induced the synthesis of $cPLA_2$ protein by stimulating $cPLA_2$ gene transcription. We observed an increase in $cPLA_2$ protein, an increase in cytosolic and membrane-bound PLA_2 activity, and an increase in total cellular PLA_2 activity after IFN-γ stimulation. However, we also observed that IFN-γ–induced $cPLA_2$ activation is not solely dependent on $cPLA_2$ synthesis. While the amount of $cPLA_2$ protein in cytosolic fraction was increased after IFN-γ stimulation for 4, 12, and 24 h, the measured PLA_2 activity in the same samples was elevated only after 4 h IFN-γ treatment. The difference between the $cPLA_2$ protein amount and the assayed PLA_2 activity may suggest that the increase in $cPLA_2$ protein requires further steps to be manifest as increased $cPLA_2$ activity. Since available evidence shows that $cPLA_2$ activity can be posttranslationally regulated in several types of cells (9–12, 41), our observations support that in the respiratory epithelium, the IFN-γ–induced $cPLA_2$ undergoes further modification of the enzyme. In Fig. 6, we found that the IFN-γ–induced $cPLA_2$ protein was not fully activated, since the enzyme activity was substantially increased by the addition of the calcium ionophore A23187. We observed that IFN-γ alone had a measurable effect on AA release over control cells at resting levels of calcium (the condition under which the time course of arachidonic acid and 15-HETE release was done in Figs. 1 and 4). A23187 caused a significantly increased release of AA from IFN-γ–treated cells compared to IFN-γ–treated cells that were not challenged with the ionophore. More importantly, IFN-γ–treated cells challenged with A23187 exhibited much more PLA_2 activity compared with control cells that were activated with A23187. Thus, there was a significant potential of enzyme activity available for activation by a sec-

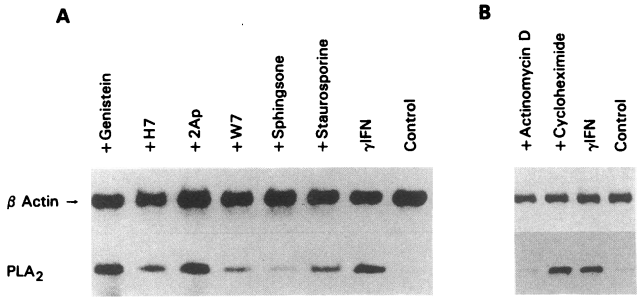

Figure 8. A representative RT-PCR experiment assessing the $cPLA_2$ mRNA level. The amplified products of β-actin (510 bp) and the corresponding products of $cPLA_2$ (306 bp) were shown. Cells were treated with IFN-γ (300 U/ml) for 4 h with or without inhibitors. Inhibitors were added to the culture 10 min before the addition of IFN-γ (300 U/ml). The effects of various protein kinase inhibitors were displayed in (*A*) from the right as following: control cells; cells treated with IFN-γ; and cells treated with IFN-γ in the presence of staurosporine (2×10^{-7} M), sphingosine (10^{-6} M), W-7 (30 μM), 2 amino purine (1 mM), H-7 (2×10^{-5} M), and genistein (0.1 mg/ml). (*B*) shows the effect of cycloheximide (40 μg/ml) and actinomycin D (10 μg/ml).

ond stimulus in the IFN-γ–treated cells compared to the control cells. Therefore, the importance of induction of protein levels of $cPLA_2$ in response to IFN-γ is that there is a greater amount of enzyme available for activation and, therefore, for production of active metabolites of arachidonic acid, as well as PAF.

The $cPLA_2$ is a novel protein induced by the IFN family. Since $cPLA_2$ plays a central role in providing AA and lysophospholipid for subsequent metabolism to prostaglandins, leukotrienes, HETEs, and PAF, potent lipid mediators of inflammation, the IFN-γ–induced synthesis and activation of $cPLA_2$ may play an important role in mediating the variety of biological functions attributed to this cytokine. The recognition of IFN-γ–induced $cPLA_2$ synthesis may also facilitate understanding the mechanisms of IFN-γ–induced signal transduction.

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