

Cross-talk between Peroxisome Proliferator-Activated Receptor δ and Cytosolic Phospholipase $A_2\alpha$ /Cyclooxygenase-2/Prostaglandin E_2 Signaling Pathways in Human Hepatocellular Carcinoma Cells

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Abstract

Peroxisome proliferator-activated receptor δ (PPAR δ) is a nuclear transcription factor that is recently implicated in tumorigenesis besides lipid metabolism. This study describes the cross-talk between the PPAR δ and prostaglandin (PG) signaling pathways that coordinately regulate human hepatocellular carcinoma (HCC) cell growth. Activation of PPAR δ by its pharmacologic ligand, GW501516, enhanced the growth of three human HCC cell lines (HuH7, HepG2, and Hep3B), whereas inhibition of PPAR δ by small interfering RNA prevented growth. PPAR δ activation up-regulates the expression of cyclooxygenase (COX)-2, a rate-limiting enzyme for PG synthesis, and tumor growth. PPAR δ activation or PGE $_2$ treatment also induced the phosphorylation of cytosolic phospholipase $A_2\alpha$ (cPLA $_2\alpha$), a key enzyme that releases arachidonic acid substrate for PG production via COX. Activation of cPLA $_2\alpha$ by the calcium ionophore A23187 enhanced PPAR δ binding to PPAR δ response element (DRE) and increased PPAR δ reporter activity, which was blocked by the selective cPLA $_2\alpha$ inhibitors. Consistent with this, addition of arachidonic acid to isolated nuclear extracts enhanced the binding of PPAR δ to DRE *in vitro*, suggesting a direct role of arachidonic acid for PPAR δ activation in the nucleus. Thus, PPAR δ induces COX-2 expression and the COX-2-derived PGE $_2$ further activates PPAR δ via cPLA $_2\alpha$. Such an interaction forms a novel feed-forward growth-promoting signaling that may play a role in hepatocarcinogenesis. (Cancer Res 2006; 66(24): 11859-68)

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide (1). The pathogenesis of hepatic carcinogenesis remains incompletely understood but is believed to involve sequential events, including chronic inflammation, hepatocyte hyperplasia, dysplasia, and, ultimately, malignant transformation (2). HCC usually develops in the presence of continuous inflammation and hepatocyte regeneration in the setting of chronic hepatitis and cirrhosis, although the molecular mechanisms linking chronic inflammation to malignant transformation remain to be further defined. Several lines of evidence suggest that mediators of

inflammation, such as prostaglandins (PG), are implicated in hepatic carcinogenesis (see ref. 3 for review). For example, increased cyclooxygenase (COX)-2 expression has been found in human and animal HCCs (4–10). Elevated levels of PGs, most notably PGE $_2$, have also been detected in HCC (11). Overexpression of COX-2 or treatment with exogenous PGE $_2$ increases human HCC cell growth and invasiveness (9, 12). The COX inhibitors, nonsteroidal anti-inflammatory drugs (NSAID), inhibit the proliferation and induce apoptosis in cultured HCC cells and in animal models of hepatocarcinogenesis (3), although these inhibitors are known to mediate effects through both COX-dependent and COX-independent mechanisms.

The first step in the formation of PGs is the liberation of arachidonic acid (5,8,11,14-eicosatetraenoic acid) from membrane-bound phospholipids, usually by the action of phospholipase enzymes, primarily phospholipase A_2 s (PLA $_2$). Although there exist multiple different isoforms of PLA $_2$ s in cells, it is the 85-kDa cytosolic PLA $_2\alpha$ (cPLA $_2\alpha$) that most commonly supplies the arachidonic acid for PG production by COX (13, 14). Two isoforms of COXs have been identified, COX-1 and COX-2, both catalyzing the conversion of arachidonic acid into endoperoxide intermediates that are ultimately converted by specific synthases to prostanoids, including PGE $_2$, the most abundant PG in human neoplastic epithelial cells (15, 16). Whereas COX-1 is constitutively expressed in most cells, COX-2 is highly induced by inflammatory cytokines/chemokines, growth factors, oncogene activation, and tumor promoters, thus contributing to the enhanced PG production when these signaling pathways are activated in inflammatory and neoplastic diseases (15, 16). PGs transduce signals mainly through binding to their specific G protein-coupled receptors along the plasma membrane. Recently, eicosanoids have been shown to regulate cell functions through activation of peroxisome proliferator-activated receptors (PPAR), which belong to the superfamily of nuclear receptors that function as ligand-activated transcription factors.

PPARs regulate gene expression by binding with their heterodimeric partner retinoid X receptor to specific peroxisome proliferator response elements. Three different PPAR subtypes have been identified: PPAR α , PPAR δ (also termed as PPAR β), and PPAR γ . PPAR α is highly expressed in liver parenchymal cells and is implicated in lipid catabolism (17–19). PPAR γ is predominantly expressed in adipose tissue and plays an important role in adipocyte differentiation, insulin sensitization, and glucose homeostasis (20, 21). PPAR β/δ shows a ubiquitous expression in most tissues (18) and is implicated in fatty acid oxidation, cell differentiation, inflammation, cell motility, and cell growth (22–32).

Recent studies suggest a potential role of PPAR δ in carcinogenesis. For example, the expression of PPAR δ is increased in

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colorectal cancer cells compared with normal colon epithelial cells (33, 34). Treatment of *Apc^{min}* mice with the PPAR δ ligand GW501516 increased the number and size of intestinal polyps (35). On the other hand, disruption of PPAR δ in human colon cancer cells by targeted homologous recombination reduced tumor growth when the PPAR $\delta^{-/-}$ cells were inoculated as xenografts in nude mice (36). These observations suggest a tumor-promoting role of PPAR δ during intestinal carcinogenesis. Additionally, PPAR δ has also been implicated in the growth of several other human cancers, including prostate and lung cell lines (37) and HCC cells (38). Activation of PPAR δ by its synthetic agonist increases COX-2 expression in human HCC cells (38). Furthermore, PPAR δ is a

downstream gene of Wnt- β -catenin signal pathway and the target of NSAIDs (33, 39). PPAR δ has also been shown to mediate the PGE $_2$ -induced intestinal adenoma growth (40). However, in spite of the documented tumor-promoting effect of PPAR δ , there is also evidence suggesting that PPAR δ might inhibit intestine tumor development (41). Thus, the precise role of PPAR δ in tumorigenesis remains to be further defined.

This study was designed to evaluate the effect and mechanism of PPAR δ in HCC cell growth. Our data show that PPAR δ promotes human HCC cell growth through up-regulation of COX-2 gene transcription and PGE $_2$ production. More importantly, the PPAR δ -induced PGE $_2$ subsequently phosphorylates and activates cPLA $_2\alpha$,

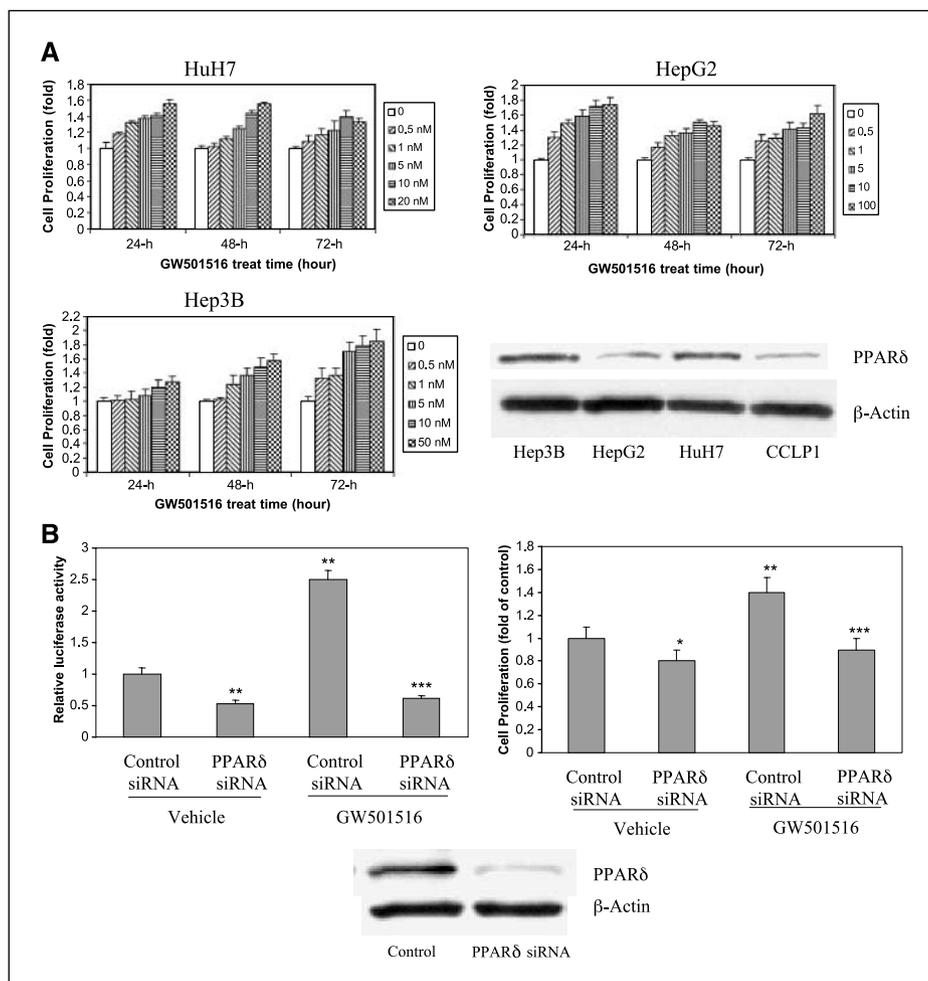


Figure 1. PPAR δ promotes human HCC cell growth and induces COX-2 gene expression. **A**, activation of PPAR δ by GW501516 promotes human HCC cell growth. HuH7, HepG2, and Hep3B cells were seeded onto 96-well plate (3,000 per well) in 10% FBS medium and incubated overnight. After 24 hours of serum starvation, GW501516 (0.5–50 nmol/L) was added into the medium and the cells were cultured for 24, 48, and 72 hours. Cell proliferation was measured using WST-1 reagent as described in Materials and Methods. *Columns*, mean of six identical treatments; *bars*, SD. The level of PPAR δ protein in human HCC cells (HuH7, HepG2, and Hep3B) was determined by SDS-PAGE and Western blot analysis (with the cholangiocarcinoma cell line CCLP1 as positive control). **B**, inhibition of PPAR δ by siRNA reduces human HCC cell growth. *Top left*, GW501516 activates PPAR δ . HuH7 cells (70% confluence in six-well plate) were cotransfected with 1 μ g of human DRE reporter plasmid and 100 pmol siRNA using LipofectAMINE 2000 reagent. After 42 hours of transfection, the cells were then treated with GW501516 (10 nmol/L) or vehicle DMSO (1:10,000 dilution) in serum-free medium for 6 hours. At the end of treatment, the cells were washed twice with cold PBS and the cell lysates were obtained for luciferase activity assay. GW501516 treatment significantly increased the DRE reporter activity, and this effect was blocked by siRNA inhibition of PPAR δ (2.5-fold versus 0.61-fold). **, $P < 0.01$, compared with control siRNA with vehicle treatment; ***, $P < 0.01$, compared with control siRNA with GW501516 treatment ($n = 3$). *Top right*, siRNA inhibition of PPAR δ prevents HCC cell growth. HuH7 cells (3,000 per well) were seeded in 96-well plate and cultured overnight. The cells were transfected overnight with 5 pmol per well of siRNA-PPAR δ or siRNA-control using LipofectAMINE 2000 reagent in 0.5% FBS medium. Following transfection, the cells were treated with GW501516 (10 nmol/L) or vehicle DMSO (1:10,000 dilution) for 24 hours. WST-1 reagent was used to determine cell growth. *Columns*, mean of three individual experiments; *bars*, SD. The cells transfected with the PPAR δ siRNA exhibited significant reduction of growth either under basal culture conditions or in response to GW501516 treatment. *, $P < 0.05$, compared with control siRNA cells without GW501516 treatment; **, $P < 0.01$, compared with control siRNA cells without GW501516 treatment; ***, $P < 0.01$, compared with control siRNA cells with GW501516 treatment. *Bottom*, PPAR δ protein levels in HuH7 cells with or without PPAR δ siRNA transfection. β -Actin was used as the loading control.

thereby providing arachidonic acid for further PPAR δ activation and PGE $_2$ production. Consequently, the interplay between PPAR δ and cPLA $_2\alpha$ /COX-2/PGE $_2$ signaling pathways forms a positive feedback loop promoting HCC cell growth.

Materials and Methods

Materials. Cell culture medium and LipofectAMINE Plus reagent were purchased from Invitrogen (Carlsbad, CA). Cell proliferation reagent WST-1 was purchased from Roche Applied Science (Indianapolis, IN). Luciferase

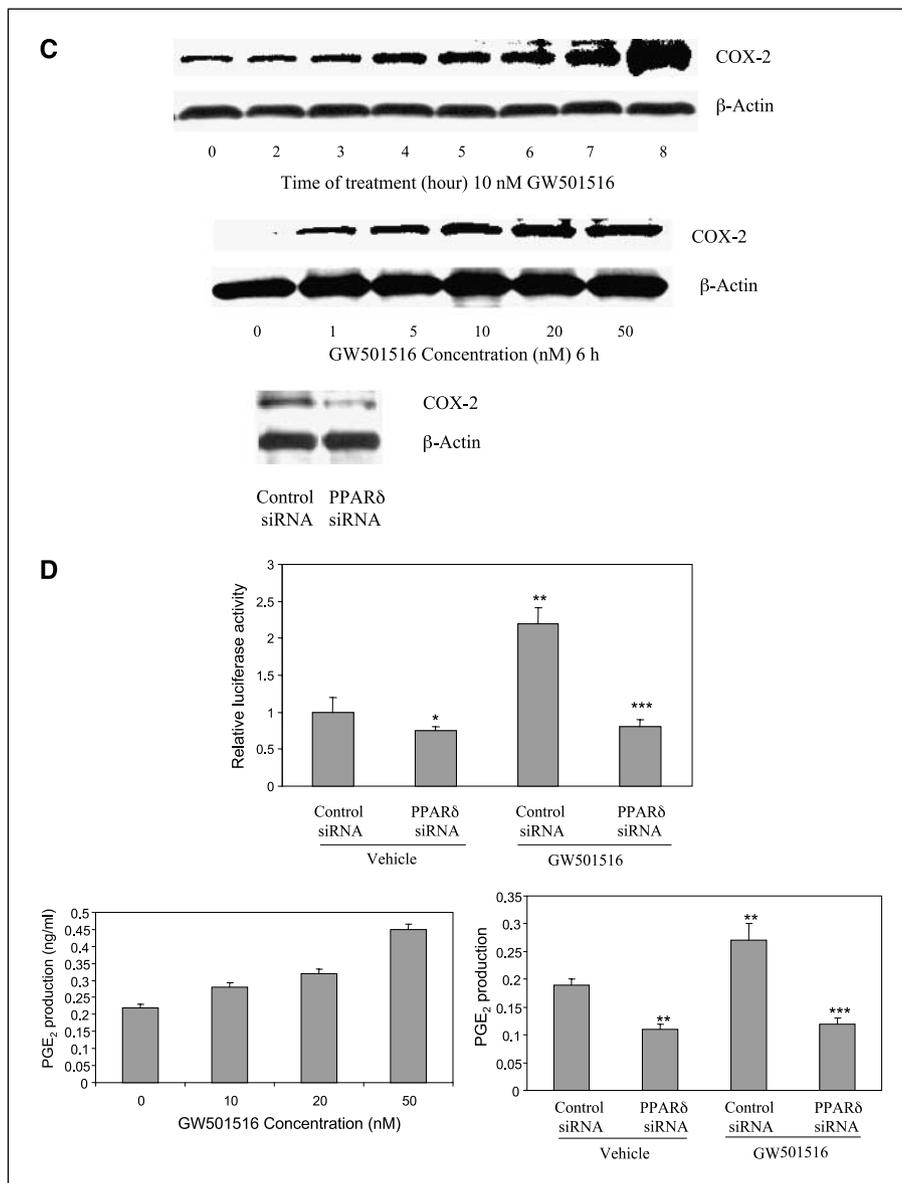


Figure 1 Continued. C, activation of PPAR δ by GW501516 enhances COX-2 protein expression. Inhibition of PPAR δ by siRNA reduces COX-2 protein level. *Top*, time course effect of GW501516 on COX-2 protein expression. HuH7 cells were pre-serum starved for 24 hours and treated with GW501516 (10 nmol/L) in serum-free medium for increasing times (2–8 hours). The cell lysates were obtained for SDS-PAGE and Western blot analysis to determine the level of COX-2 protein. The same blot was stripped and reprobed with β -actin antibody as loading control. Increased COX-2 protein was observed after GW501516 treatment for 4 to 8 hours. *Middle*, dose-dependent effect of GW501516 on COX-2 protein expression. HuH7 cells were incubated with GW501516 (1–50 nmol/L) in serum-free medium for 6 hours, and the whole-cell lysates were obtained for SDS-PAGE and Western blot analysis to determine the level of COX-2 protein. *Bottom*, siRNA inhibition of PPAR δ reduces COX-2 protein level. HuH7 cells (70% confluence) were transfected with PPAR δ siRNA or nontargeted control siRNA for 48 hours. Cellular protein (30 μ g) was subjected to SDS-PAGE and Western blotting to determine COX-2 protein, with β -actin as loading control. *D*, activation of PPAR δ by GW501516 enhances COX-2 gene promoter activity and PGE $_2$ production. *Top*, GW501516 treatment enhanced COX-2 gene promoter reporter activity. HuH7 cells (70% confluence in six-well plate) were cotransfected with the COX-2 promoter reporter plasmid (1 μ g) plus control siRNA or PPAR δ siRNA using LipofectAMINE 2000 reagent. After 48 hours, the cells were incubated with GW501516 (10 nmol/L) or vehicle DMSO (1:10,000 dilution) for 6 hours. Following washing twice with cold PBS, the cells were lysed and the cell lysates were obtained for luciferase activity assay. GW501516 treatment (10 nmol/L) induced a 2.2-fold increase of the COX-2 promoter reporter activity. **, $P < 0.01$ ($n = 3$). This effect was completely blocked by siRNA inhibition of PPAR δ . ***, $P < 0.01$, compared with control siRNA plus GW501516 treatment ($n = 3$). PPAR δ siRNA also inhibited the COX-2 promoter activity in cells without GW501516 treatment. *, $P < 0.05$, compared with control siRNA with vehicle treatment. *Bottom left*, GW501516 treatment increases PGE $_2$ production. HuH7 cells (80% confluence in six-well plate, with overnight serum starvation) were treated with GW501516 (10–50 nmol/L) or vehicle for 6 hours in serum-free medium. At the end of treatment, the culture medium was collected and cell debris was removed by centrifugation for 25 minutes at 4°C. Medium (100 μ L) was used for the measurement of PGE $_2$. *Columns*, mean of three experiments (0.22 ng/mL for control versus 0.28, 0.32, and 0.45 ng/mL for 10, 20, and 50 nmol/L GW501516, respectively); *bars*, SD. *Bottom right*, siRNA inhibition of PPAR δ prevents GW501516-induced PGE $_2$ production. HuH7 cells were transfected with PPAR δ siRNA or control siRNA for 48 hours followed by vehicle DMSO or GW501516 treatment for 6 hours. **, $P < 0.01$, compared with control siRNA plus vehicle treatment; ***, $P < 0.01$, compared with control siRNA plus GW501516 treatment ($n = 3$).

Assay System and reporter lysis buffer were from Promega Corp. (Madison, WI). Antibody providers are as follows: anti-COX-2 (Cayman Chemical Co., Ann Arbor, MI); anti-cPLA₂, anti-PPAR δ , and anti-epidermal growth factor receptor (EGFR; Santa Cruz Biotechnology, Santa Cruz, CA); anti-phosphorylated cPLA₂ α (Ser⁵⁰⁵) and Akt Kinase Assay kit (Cell Signaling Technology, Danvers, MA); anti-phosphorylated EGFR (BD Biosciences, San Jose, CA); and anti- β -actin (Sigma, St. Louis, MO). Chemiluminescence detection reagent was from Amersham Biosciences (Piscataway, NJ). PPAR δ agonist GW501516 was purchased from Cayman Chemical. PGE₂, indomethacin, arachidonic acid, A23187, the cPLA₂ inhibitors pyrrolidine derivatives and AACOCF₃, the EGFR tyrosine kinase inhibitor AG1478, the p38 kinase inhibitor SB203580, the protein kinase C (PKC) inhibitor bisindolylmaleimide I, the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, and the p44/42 mitogen-activated protein kinase (MAPK) inhibitor PD98059 were purchased from Calbiochem (San Diego, CA). The PGE₂ enzyme immunoassay system was purchased from Amersham Biosciences. The PPAR δ Transcription Factor Assay kit was from Cayman Chemical. The nuclear extraction kit was purchased from Sigma. Small interfering RNA (siRNA)-PPAR δ , siRNA-COX-2, and siRNA-control were from Dharmacon, Inc. (Lafayette, CO). The 5'-biotinylated PPAR δ response element (DRE) oligonucleotides were synthesized by Sigma-Genosys (Woodland, TX), and the unlabeled DRE oligonucleotides were from Integrated DNA Technologies, Inc. (Coralville, IA). The immobilized streptavidin beads were purchased from Pierce (Rockford, IL). Poly(dI-dC).poly(dI-dC) was from Amersham Biosciences.

Cell culture and proliferation assay. Three hepatocarcinoma cell lines, HuH7, HepG2, and Hep3B, were cultured in Eagle's Minimum Essential Medium with 10% fetal bovine serum (FBS). Cell growth was determined using the cell proliferation reagent WST-1, which is a tetrazolium salt cleaved by mitochondrial dehydrogenases in viable cells. Briefly, the cells (3,000 per well) were seeded on 96-well plate and incubated at 37°C overnight. The cells were then treated with GW501516 or transfected with siRNAs for indicated times. WST-1 (10 μ L) was subsequently added to each well, and the culture was continued for 30 minutes to 4 hours before measurement of A_{450 nm} using an automatic ELISA plate reader.

Transient transfection and luciferase reporter assay. Cells were seeded in six-well plate in culture medium containing 10% FBS the day before transfection. On the following day, the cells in each well (70–80% confluence) were transfected with 1 μ g of plasmid using LipofectAMINE Plus reagent (Plus reagent, 6 μ L; LipofectAMINE, 4 μ L) or with siRNA using Lipofect-AMINE 2000 reagent in serum-free medium. After 3 hours of transfection, the transfection medium was replaced with culture medium containing 10% FBS. At the end of indicated treatment, the cells were washed twice in ice-cold PBS and lysed with reporter lysis buffer on ice for 20 minutes. The cells were then scraped down and spun at 14,000 rpm for 10 minutes in cold room. The supernatant was collected for luciferase activity assay using a Berthold AutoLumat LB 953 luminometer (Berthold, Nashua, NH).

Preparation of whole-cell lysate and immunoblotting. HuH7 cells were grown on six-well plates and treated with different concentration of GW501516 or other indicated reagents in FBS-free medium. The vehicle, DMSO, was added to the control culture. Following treatment for indicated times, the cells were washed twice with cold PBS and scraped down. The cell pellets were washed two more times with cold PBS and then resuspended in homogenization buffer containing 50 mmol/L HEPES (pH 7.55), 1 mmol/L EDTA, 1 mmol/L DTT, and 1 mmol/L mammalian protease inhibitor cocktail (Sigma). The cell suspension was placed on ice and sonicated four times for 15 seconds. The samples were then centrifuged at 14,000 rpm for 10 minutes at 4°C, and the supernatants were collected as whole-cell lysate. Total protein concentration was measured by bicinchoninic acid (BCA) reagent (Pierce). Cell lysate was aliquoted and frozen at –80°C until use. For immunoblotting, 20 to 35 μ g protein was separated on 4% to 20% Tris-glycine gels and the separated proteins were electrophoretically transferred onto the nitrocellulose membrane (Bio-Rad, Hercules, CA). Nonspecific binding was blocked with 5% nonfat milk dissolved in 0.5% Tween 20 in PBS (PBST) for 1 hour at room temperature. The membrane was then incubated overnight with primary antibodies (1:1,000 dilution for COX-2, EGFR, phosphorylated EGFR, Akt, phosphorylated Akt, and β -actin;

1:2,000 dilution for PPAR δ) in 5% milk PBST. Following repeated washing with PBST the next day, the membranes were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000 dilution) for 1 hour at room temperature. After washing, the blots were developed using the enhanced chemiluminescence (ECL) Western blotting detection system and exposed to Eastman Kodak (Rochester, NY) MR radiographic films.

Immunoprecipitation and Western blotting for cPLA₂ α phosphorylation. To immunoprecipitate cPLA₂ α , 300 μ L of whole HuH7 cell lysate (~50 μ g protein) in a 1.5 mL Eppendorf tube were precleared with 20 μ L protein A/G agarose (Santa Cruz Biotechnology) for 1 hour at 4°C. The cleared cell lysate was then incubated with 5 μ L mouse anti-human cPLA₂ α monoclonal antibody at 4°C for 3 hours with gentle agitation. Protein A/G agarose (20 μ L) was then added, and the sample was kept at 4°C for 16 hours, with gentle agitation, to precipitate cPLA₂ α -antibody complex. The protein A/G agarose pellet was collected by centrifuge and washed four times with cold homogenization buffer at 4°C. SDS sample loading buffer (20 μ L) was then added to the pellet, and the mixture was boiled for 5 minutes before SDS-PAGE using 4% to 20% Tris-glycine gels. After blocking nonspecific binding, the blot was incubated overnight with rabbit anti-phosphorylated cPLA₂ α (Ser⁵⁰⁵) antibody (1:1,000 dilution) in 5% milk PBST at 4°C. The HRP-conjugated donkey anti-rabbit antibody (1:10,000 dilution) was used as the second antibody. Specific cPLA₂ α band was visualized by ECL Western blotting detection system.

Measurement of PGE₂ production. HuH7 cells cultured in serum-free medium in six-well plates were treated as indicated in the text. The supernatant was collected and centrifuged to remove floating cells. Each sample (100 μ L) was used to measure PGE₂ level using the PGE₂ enzyme immunoassay system as described previously (9, 42).

Purification of nuclear extract. HuH7 cells cultured in 100-mm dishes at 80% to 90% confluence were treated as described in the text. Following treatment, the cells were washed twice with ice-cold PBS and scraped with a rubber policeman. The cell pellet was then swelled in 5-fold volume of hypotonic buffer for 20 minutes on ice. Following homogenization using 27-gauge sterile needle on ice, the nuclei were pelleted by centrifugation at 600 \times g for 10 minutes. The nuclei were then washed twice in the isotonic buffer and resuspended in HKMG buffer [10 mmol/L HEPES, (pH 7.9), 100 mmol/L KCl, 5 mmol/L MgCl₂, 10% glycerol, 1 mmol/L DTT, 0.5% NP40] containing protease inhibitors and phosphatase inhibitors. The nuclei suspension was then subjected to sonication, and the cellular debris was removed by centrifugation at 14,000 rpm for 20 minutes at 4°C. The supernatant was collected as nuclear extract and frozen at –80°C until use. Aliquots of the nuclear extracts were used to quantitate the protein concentration using the BCA reagent.

ELISA-based PPAR δ binding to its DNA response element. The experiments were carried out using the 96-well ELISA kit purchases from Cayman Chemical. Briefly, the oligonucleotide containing the PPAR δ binding consensus sequence was immobilized onto the bottom of wells. Nuclear extract (50 μ g) from treated cells or control cell was added to the dsDNA-coated well and incubated at 4°C overnight. After complete washing, PPAR δ antibody was added and the samples were incubated at room temperature for 1 hour. The HRP-conjugated secondary antibody and developing solution were sequentially added, and the A_{655 nm} value was determined.

Biotinylated DRE oligonucleotide precipitation assay. The assay was done as previously reported with modification (43). The nucleotide sequences of biotinylated DRE were 5'-GCGTGAGCGCTCACAGGT-CAATTCG-3' and 5'-CCGAATTGACCTGTGAGCGCTCACG-3' (33). These two complementary strands were annealed in TEN buffer. After treatment, the cells were lysed by sonication in 200 μ L HKMG buffer containing protease and phosphatase inhibitors. The cellular debris was removed by centrifugation. The cell extracts (50 μ g) were precleared with 30 μ L immobilized streptavidin-agarose beads for 1 hour at 4°C with gentle agitation. The cleared nuclear extracts were then incubated with 1 μ g of biotinylated double-strand DRE and 10 μ g of poly(dI-dC).poly(dI-dC) for 16 hours. DRE-bound protein was pulled down by incubating the samples with 35 μ L of streptavidin-agarose beads for 1 hour at 4°C with gentle agitation. The agarose mixture was collected by centrifugation and washed four times with cold HKMG buffer. SDS sample buffer was then added to

the pellet. The samples were boiled for 5 minutes and subjected to SDS-PAGE and Western blotting for PPAR δ .

Statistical analysis. Statistical analysis was done using Microsoft Excel 2003 software. Comparisons were done using two-tailed unpaired Student's *t* test. Values of *P* < 0.05 were considered statistically significant.

Results and Discussion

The role of PPAR δ in human HCC cell growth was first examined by using GW501516, a synthetic pharmacologic ligand that is selective for PPAR δ with no effect on PPAR α or PPAR γ (even at dose as high as 10 μ mol/L; refs. 22, 35, 44). As shown in Fig. 1A, GW501516 treatment increased the growth of three human HCC

cell lines (HuH7, HepG2, and Hep3B). This effect was dose dependent (0.5–100 nmol/L) and was observed at different treatment periods (24–72 hours). The dose range is lower than that used in a previous study (38), possibly due to the difference in compound source and quality. Western blot analysis reveals that PPAR δ protein is expressed in all these cells (Fig. 1A). The transcriptional function of PPAR δ in these cells was verified by the observation that GW501516 treatment significantly increased the reporter activity of a luciferase promoter construct containing the DRE (33). Accordingly, inhibition of PPAR δ by siRNA blocked the DRE activity in cells with or without GW501516 treatment (Fig. 1B). The direct effect of PPAR δ on HCC growth was further

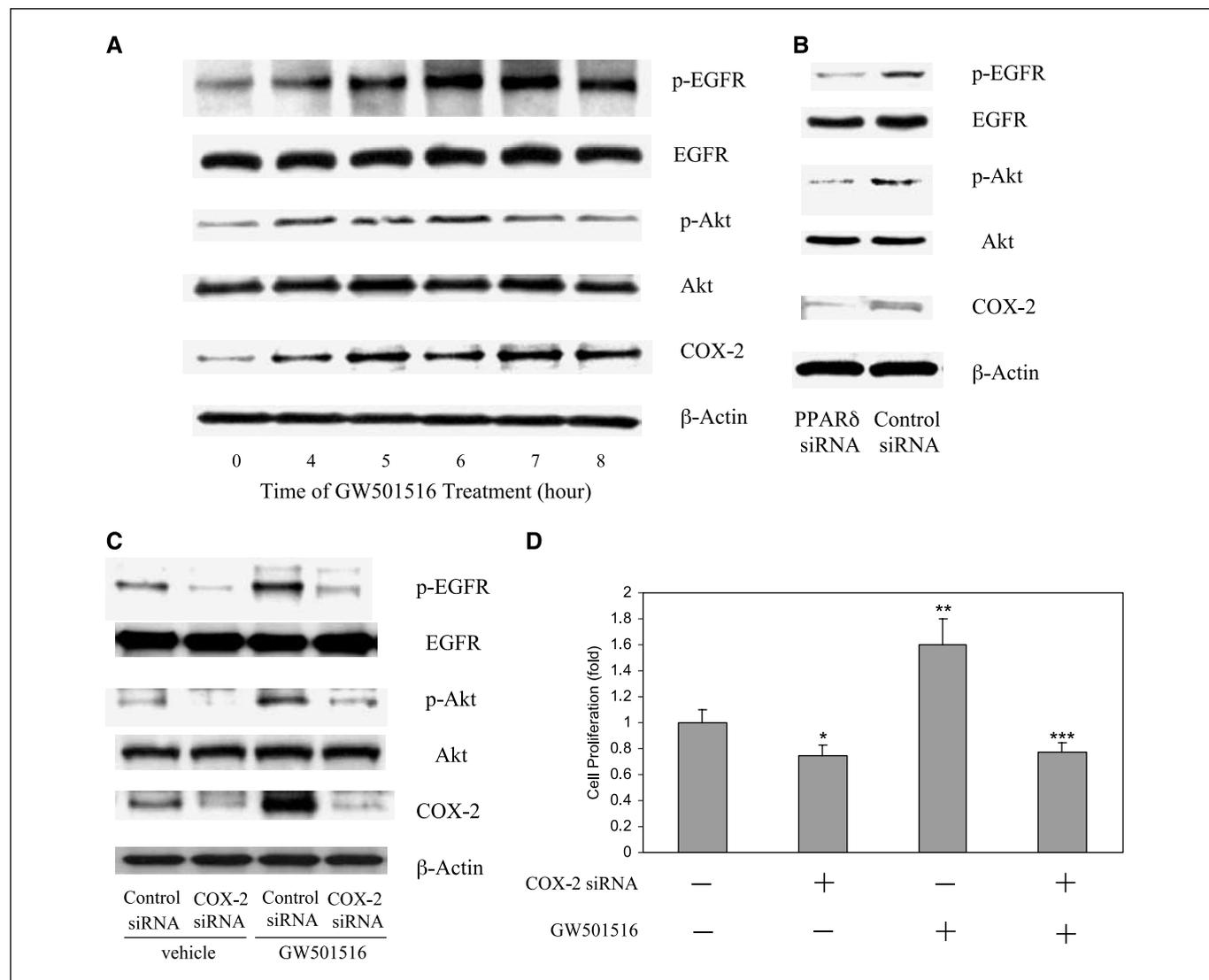


Figure 2. Activation of PPAR δ by GW501516 induces the phosphorylation of EGFR and Akt through COX-2 in HCC cells. **A**, GW501516 induces EGFR and Akt phosphorylation. HuH7 cells (80% confluence in six-well plate with overnight serum starvation) were treated with GW501516 (10 nmol/L) for different times (4–8 hours). Whole-cell lysates were collected, and 30 μ g protein was subjected to Western blotting for detection of phosphorylated EGFR (p-EGFR), EGFR, phosphorylated Akt (p-Akt), Akt, COX-2, and β -actin. GW501516 treatment increased the level of COX-2 expression as well as the phosphorylation of EGFR and Akt. **B**, siRNA inhibition of PPAR δ reduces phosphorylated EGFR and phosphorylated Akt levels. HuH7 cells were transfected with control or PPAR δ siRNA for 48 hours. Cellular protein (30 μ g) was subjected to SDS-PAGE and Western blotting. **C**, siRNA inhibition of COX-2 prevents GW501516-induced EGFR and Akt phosphorylation. HuH7 cells (90% confluence in six-well plate) were transfected with COX-2 siRNA or nontargeted control siRNA using LipofectAMINE 2000 reagent for 48 hours followed by treatment with GW501516 (10 nmol/L) or vehicle DMSO (1:10,000) for 6 hours. Whole-cell lysate (30 μ g) was subjected to Western blotting for detection of COX-2, phosphorylated EGFR, EGFR, phosphorylated Akt, Akt, and β -actin. All the experiments were repeated thrice. **D**, inhibition of COX-2 by siRNA prevents GW501516-induced cell growth. HuH7 cells transfected with control siRNA or COX-2 siRNA for 24 hours were treated with GW501516 or vehicle for additional 24 hours. Cell proliferation assay was carried out using WST-1 assay. *, *P* < 0.05, compared with control siRNA plus vehicle treatment; **, *P* < 0.01, compared with control siRNA plus vehicle treatment; ***, *P* < 0.01, compared with control siRNA plus GW501516 treatment.

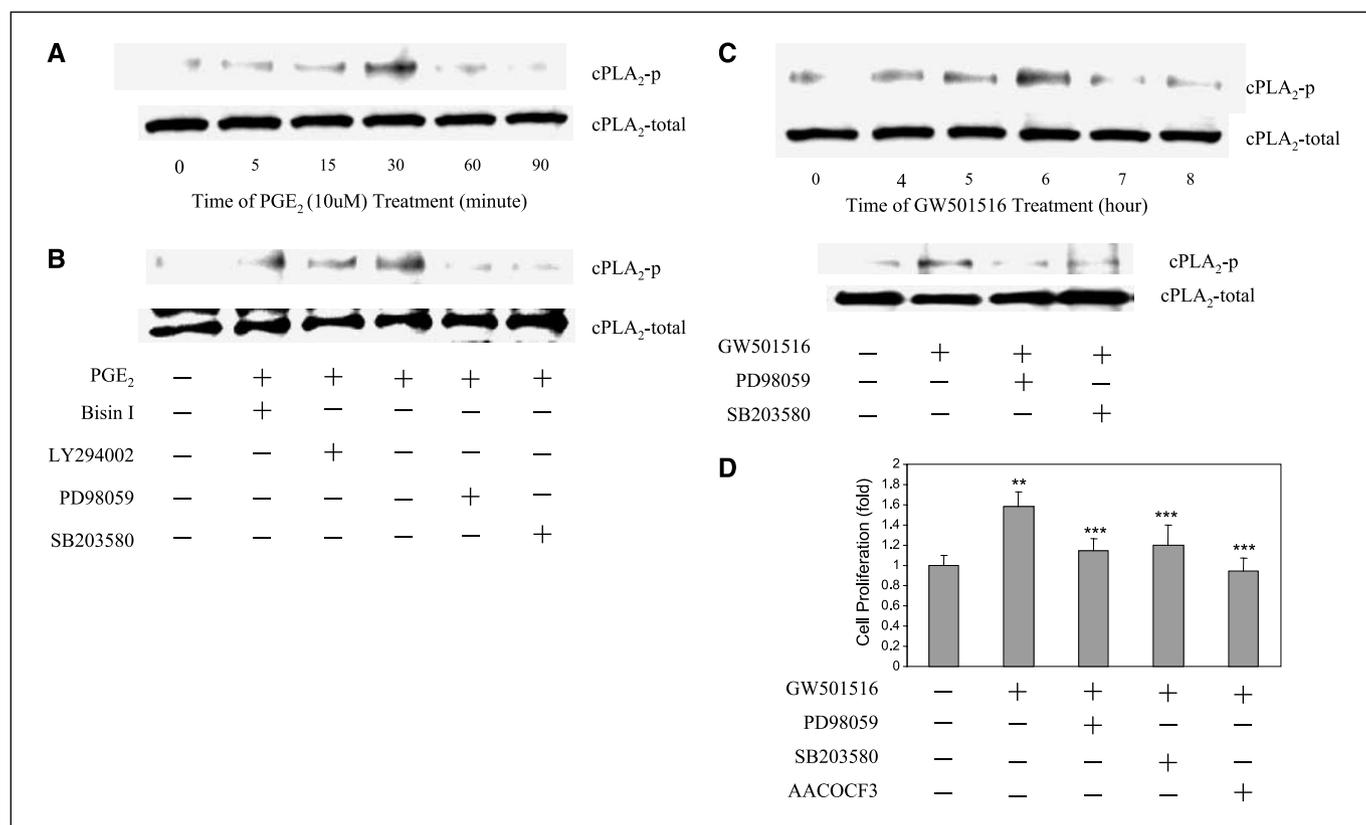


Figure 3. GW501516 and PGE₂ increase the phosphorylation of cPLA₂α in human HCC cells. Evidence for involvement of p42/44 and p38 MAPKs. **A**, PGE₂ treatment enhances cPLA₂α phosphorylation. HuH7 cells (80% confluence in six-well plate with overnight serum starvation) were incubated with PGE₂ (10 μmol/L) for 5 to 90 minutes. The whole-cell lysates were then collected and precleared with protein A/G agarose beads for 1 hour followed by overnight incubation with cPLA₂α antibody at 4 °C. The protein-antibody complex was pulled down by incubation with protein A/G agarose beads for 1 hour and subjected to Western blotting for detection of phosphorylated cPLA₂α (cPLA₂-p) and total cPLA₂α (cPLA₂-total). A similar effect was also seen in HepG2 cells. **B**, inhibition of p42/44 and p38 MAPKs prevents PGE₂-induced cPLA₂α phosphorylation. HuH7 cells (80% confluence in six-well plate) were serum starved overnight and preincubated for 30 minutes with PKC inhibitor [bisindolylmaleimide I (*Bisin I*), 20 μmol/L], PI3K inhibitor (LY294002, 20 μmol/L), p44/42 inhibitor (PD98059, 20 μmol/L), p38 inhibitor (SB203580, 10 μmol/L), or vehicle DMSO (1:10,000 dilution) followed by coincubation with PGE₂ (10 μmol/L) for additional 30 minutes. The whole-cell lysates were collected and subjected to immunoprecipitation and Western blot analysis to determine cPLA₂α phosphorylation. The PGE₂-induced cPLA₂α phosphorylation was blocked by the p44/42 MAPK inhibitor PD98059 or the p38 MAPK inhibitor SB203580 but not by the PKC inhibitor bisindolylmaleimide I or the PI3K inhibitor LY294002. **C**, GW501516 treatment increases cPLA₂α phosphorylation. This effect is inhibited by the MAPK inhibitors. *Top*, effect of GW501516 on cPLA₂α phosphorylation. HuH7 cells (80% confluence in six-well plate) were serum starved overnight and then incubated with GW501516 (10 nmol/L) for 4 to 8 hours. Whole-cell lysates were collected and subjected to immunoprecipitation and Western blot to detect cPLA₂α phosphorylation. GW501516 treatment increased cPLA₂α phosphorylation, with peak effect at 6 hours. All the experiments were repeated thrice. *Bottom*, MAPK inhibitors prevent GW501516-induced cPLA₂α phosphorylation. HuH7 cells were treated with GW501516 (10 nmol/L) for 6 hours in the presence or absence of PD98059 (20 μmol/L) or SB203580 (10 μmol/L). **D**, inhibitors of MAPKs and cPLA₂α prevent GW501516-induced cell growth. HuH7 cells were cotreated overnight with GW501516 plus vehicle (DMSO, 1:10,000), PD98059 (20 μmol/L), SB203580 (10 μmol/L), or AACOCF₃ (25 μmol/L). WST-1 reagents were used for cell proliferation assay. **, *P* < 0.01, compared with vehicle; ***, *P* < 0.05, compared with GW501516 treatment (*n* = 6).

supported by the observation that siRNA inhibition of PPARδ significantly reduced the growth of human HCC cells, both under spontaneous culture and in response to GW501516 treatment (Fig. 1B). These results document an important role of PPARδ in human HCC cell growth.

Because COX-2-derived PGE₂ signaling is implicated in hepatocarcinogenesis, we postulated that COX-2 and PGE₂ signaling might play a role in PPARδ-induced HCC cell growth. To evaluate this hypothesis, we examined the effect of PPARδ activation on COX-2 gene expression in HCC cells. As shown in Fig. 1C, activation of PPARδ by GW501516 enhanced the level of COX-2 protein in HuH7 cells, whereas siRNA inhibition of PPARδ reduced it (a similar effect was also observed in HepG2 cells). This effect is likely mediated through up-regulation of COX-2 gene transcription because GW501516 treatment enhanced the COX-2 promoter reporter activity (Fig. 1D). Accordingly, GW501516 treatment also induced a dose-dependent increase of PGE₂ production, which was blocked by siRNA inhibition of PPARδ (Fig. 1D). These findings

show a stimulatory effect of PPARδ on COX-2 expression and PGE₂ production in human HCC cells. Our data are consistent with a recent study showing that PPARδ agonist GW501516 enhances COX-2 gene reporter activity in HepG2 cells (38). The exact mechanism for PPARδ-induced COX-2 expression is not fully understood at the present time. Because the DRE is not identified in human COX-2 promoter, it is possible that PPARδ may regulate COX-2 gene transcription indirectly through control of other transcriptional factors, although the possibility of PPARδ binding to other unidentified DNA element in the COX-2 gene cannot be entirely excluded.

Given that COX-2-derived PGE₂ has been shown to promote HCC cell growth through activation of EGFR and Akt (9, 12), we next determined the potential effect of PPARδ activation on EGFR and Akt phosphorylation. As shown in Fig. 2A and B, activation of PPARδ by GW501516 (10 nmol/L) enhanced EGFR and Akt phosphorylation, whereas inhibition of PPARδ decreased their phosphorylation. Furthermore, siRNA inhibition of COX-2

prevented both endogenous and GW501516-induced EGFR and Akt phosphorylation (Fig. 2C). Consistent with these observations, COX-2 siRNA also inhibits cell growth both under basal culture condition and with GW501516 treatment (Fig. 2D). The above results further support the involvement of COX-2 in PPAR δ -mediated EGFR/Akt phosphorylation and cell growth.

cPLA $_2\alpha$ is the rate-limiting enzyme that releases arachidonic acid from membrane phospholipids and thus provides substrate for COX enzymes. The cPLA $_2\alpha$ - and COX-2-controlled PG synthesis has been implicated in hepatocarcinogenesis (3). Whereas coupled activation of cPLA $_2\alpha$ and COX-2 plays an important role for PG production (45–47), there is also evidence indicating that PGE $_2$ can further activate cPLA $_2\alpha$ in other cancer cells (48). Therefore, we sought to further determine whether PPAR δ -induced PGE $_2$ synthesis might affect cPLA $_2\alpha$ activation in human HCC cells. As shown in Fig. 3A, treatment of HCC cells with 10 μ mol/L PGE $_2$ for 30 minutes increased the phosphorylation of cPLA $_2\alpha$. This effect was completely blocked by pretreatment with the p44/42 MAPK

inhibitor (PD98059, 20 μ mol/L) or the p38 MAPK (SB203580, 10 μ mol/L) but not by the PKC inhibitor (bisindolylmaleimide I, 20 μ mol/L) or the PI3K/Akt inhibitor (LY294002, 20 μ mol/L; Fig. 3B). These observations suggest the involvement of p38 and p42/44 MAPKs in PGE $_2$ -induced cPLA $_2\alpha$ phosphorylation in HCC cells.

Consistent with the effect of PPAR δ on COX-2 expression and PGE $_2$ synthesis, activation of PPAR δ by GW501516 also increased cPLA $_2\alpha$ phosphorylation in HCC cells, which was blocked by the p38 and p42/44 MAPK inhibitors (Fig. 3C). The GW501516-induced cPLA $_2\alpha$ phosphorylation was observed at 6 hours, whereas the GW501516-induced COX-2 protein increase was observed \sim 4 hours after treatment, and the effect peaks at 8 hours (Fig. 1C). The reason for lacking cPLA $_2\alpha$ phosphorylation at 8-hour time point is unclear and remains speculative, although the possibility of phosphatase involvement cannot be excluded.

These results presented in the above sections suggest that PPAR δ induces COX-2 expression and PGE $_2$ production that in turn

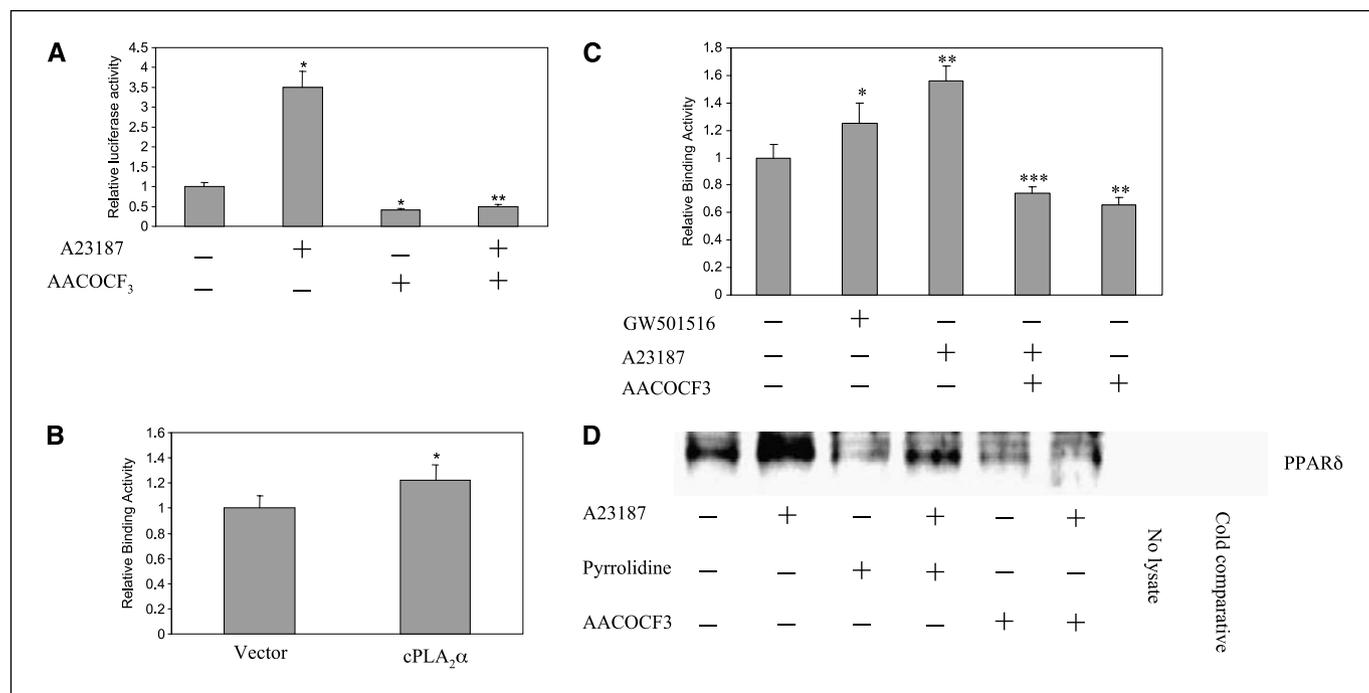


Figure 4. cPLA $_2\alpha$ enhances PPAR δ activation in human HCC cells. **A**, effect of cPLA $_2\alpha$ on DRE reporter activity. HuH7 cells (90% confluence in six-well plate) transfected with 1 μ g of DRE-driven luciferase reporter plasmid were pretreated with AACOCF $_3$ (25 μ mol/L) or vehicle DMSO (1:10,000 dilution) for 8 hours. The cells were then exposed to A23187 (1 μ mol/L) for 20 minutes, and the cultures were continued for additional 2 hours. At the end of incubation, the cells were washed twice with cold PBS and the cell lysates were obtained to measure luciferase reporter activity. A23187 treatment induced a 3.5-fold increase of the DRE reporter activity. *, $P < 0.01$, compared with control ($n = 3$). This effect was completely blocked by pretreatment with AACOCF $_3$. **, $P < 0.01$, compared with A23187 treatment ($n = 3$). Treatment with AACOCF $_3$ also significantly inhibited basal level of DRE reporter activity. *, $P < 0.01$, compared with control ($n = 3$). **B**, overexpression of cPLA $_2\alpha$ enhances PPAR δ binding to its response element (ELISA-based PPAR δ transcription factor assay). HuH7 cells were transfected with the cPLA $_2\alpha$ expression plasmid or the control vector pMT-2, and the nuclear extracts were obtained. Nuclear extract (50 μ g) was added into PPAR response element-coated 96-well plate and incubated overnight to allow protein-DNA binding. After complete wash, anti-PPAR δ antibody was added into sample wells and the plates were incubated for 1 hour. For color development, HRP-conjugated secondary antibody and color development solution were added and $A_{655 \text{ nm}}$ value was measured. Columns, mean of three experiments; bars, SD. *, $P < 0.05$. **C**, activation of cPLA $_2\alpha$ enhances PPAR δ binding to its response element (ELISA-based PPAR δ transcription factor assay). HuH7 cells were serum starved overnight and then treated with GW501516 (10 nmol/L, 8 hours) or AACOCF $_3$ (25 μ mol/L, 8 hours) before the addition of A23187 (1 μ mol/L, 20 minutes). Nuclear extract (50 μ g) from these cells was used for PPAR δ transcription factor assay as described above. Columns, mean of three individual experiments; bars, SD. *, $P < 0.05$, compared with vehicle; **, $P < 0.01$, compared with vehicle; ***, $P < 0.01$, compared with A23187. **D**, activation of cPLA $_2\alpha$ promotes PPAR δ binding to DRE (biotinylated DRE oligonucleotide precipitation assay). HuH7 cells (with overnight serum starvation) were treated with the cPLA $_2\alpha$ inhibitors AACOCF $_3$ (25 μ mol/L, 8 hours) and the trisubstituted pyrrolidine derivative (2 μ mol/L, 20 minutes) or vehicle DMSO (1:10,000 dilution) before the addition of the calcium ionophore A23187 (1 μ mol/L, 20 minutes). At the end of treatment, the cell lysates were obtained and processed for biotinylated DRE oligonucleotide precipitation assay. The cell lysates (\sim 50 μ g per sample) were precleared with immobilized streptavidin-agarose beads for 1 hour at 4°C followed by incubation overnight with biotin-labeled DRE oligonucleotide. The protein-DRE complex was pulled down by incubation with immobilized streptavidin beads for 1 hour at 4°C, and the samples were subjected to Western blotting to detect bound PPAR δ as described in Materials and Methods. Activation of cPLA $_2$ by A23187 enhanced the binding of PPAR δ to DRE. Both cPLA $_2\alpha$ inhibitors AACOCF $_3$ and the trisubstituted pyrrolidine derivative inhibited the spontaneous or A23187-induced binding of PPAR δ to DRE. The binding specificity was verified by the absence of binding in cold competitive control (biotinylated DRE oligonucleotide admixed with 100-fold of unlabeled DRE oligonucleotide) or with omission of cell lysate. All the experiments were repeated thrice.

enhances cPLA₂α phosphorylation, thus further amplifying PGE₂ signaling. The involvement of COX-2 and cPLA₂α in PPARδ-mediated cell growth is further supported by the observation that inhibiting COX-2 or cPLA₂α activation prevents PPARδ agonist-induced HCC cell growth (Figs. 2D and 3D).

Although recent evidence suggests the involvement of cPLA₂ in the activation of PPARα and PPARγ in primary and transformed hepatocytes and lung epithelial cells (49, 50), the potential role of cPLA₂ in PPARδ activation has not been investigated. In this study, the effect of cPLA₂ on PPARδ activation was examined in human HCC cells. To this end, HuH7 cells transfected with the DRE reporter plasmid (a luciferase reporter construct under the control of DRE) were treated with the calcium ionophore A23187. The use of A23187 was based on the fact that calcium is required for the nuclear translocation and activation of cPLA₂α. As shown in Fig. 4A, activation of cPLA₂α by A23187 (1 μmol/L) significantly

increased the PPARδ transcription activity in HuH7 cells (3.5-fold of control; $P < 0.01$); this effect was inhibited by the cPLA₂ inhibitor AACOCF₃ (25 μmol/L). In addition, inhibition of cPLA₂ by AACOCF₃ also decreased basal level of PPARδ transcription activity. These findings suggest the involvement of cPLA₂ in PPARδ activation.

The role of cPLA₂α in PPARδ activation was further examined by assessing the binding of PPARδ to DRE *in vitro*. For this purpose, two complementary approaches were used, including the biotinylated oligonucleotide precipitation assay to characterize the specific binding phenomenon and the ELISA-based nuclear transcription factor assay to quantitate the amount of PPARδ bound to its response element. As shown in Fig. 4B, overexpression of cPLA₂α significantly increased the binding of PPARδ to its response element as determined by the ELISA-based nuclear transcription factor assay. Furthermore, activation of cPLA₂ by

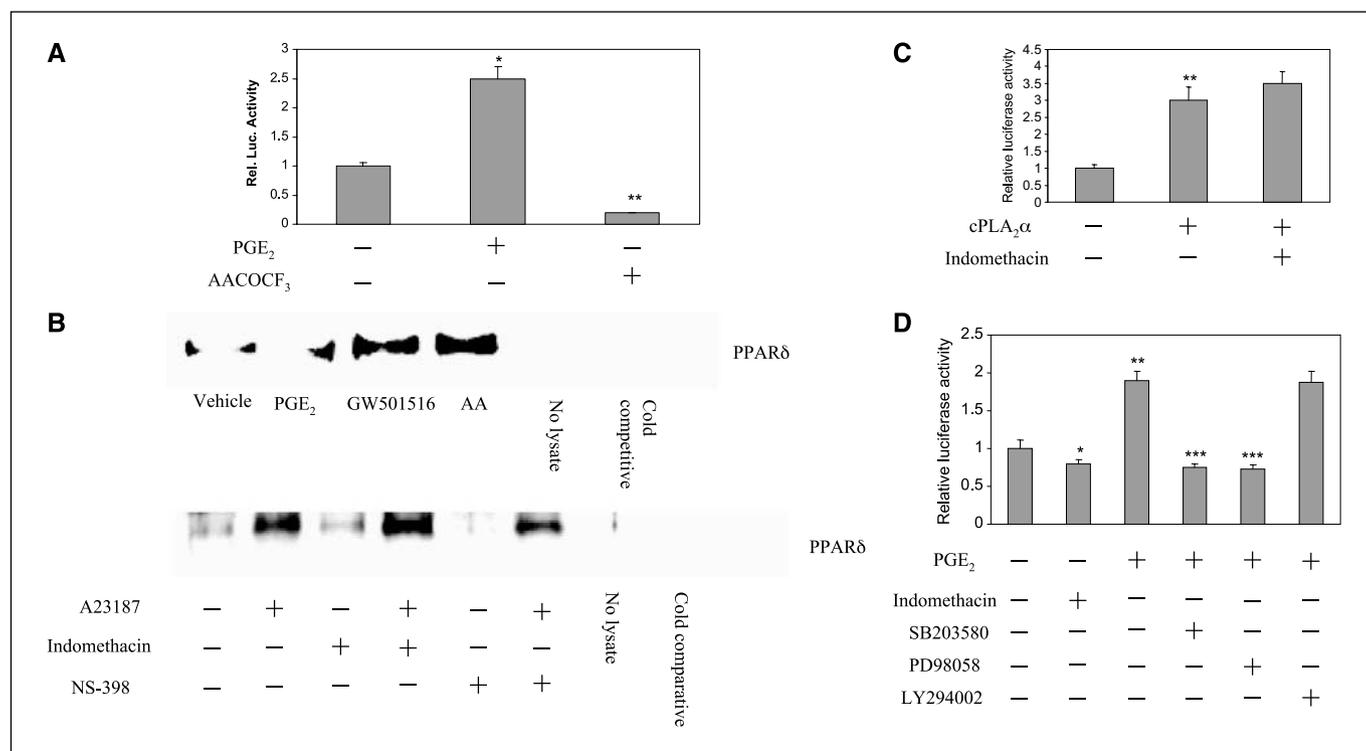
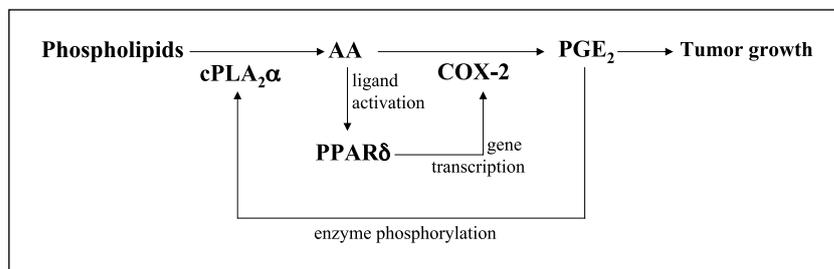


Figure 5. The effect of arachidonic acid versus PGE₂ on PPARδ activation in human HCC cells. **A**, cPLA₂ activity is required for PGE₂-induced PPARδ activation in HCC cells. HuH7 cells (90% confluence in six-well plate) were transfected overnight with 1 μg of DRE reporter plasmid using LipofectAMINE Plus reagent. Following transfection, the cells were incubated with AACOCF₃ (25 μmol/L) for 4 hours followed by treatment with PGE₂ (10 μmol/L) for additional 4 hours. The cells were then washed twice with cold PBS, and the cell lysates were obtained for luciferase activity assay. PGE₂ treatment induced a 2.5-fold increase of the DRE reporter activity, which was completely blocked by pretreatment with AACOCF₃. *, $P < 0.01$, compared with vehicle control; **, $P < 0.01$, compared with PGE₂ treatment ($n = 3$). **B**, direct effect of arachidonic acid on PPARδ binding to DRE. *Top*, addition of arachidonic acid (AA) to isolated nuclear extract induces PPARδ binding to DRE *in vitro*. The nuclear extract isolated from serum-starved HuH7 cells was precleared for 1 hour by incubation with immobilized streptavidin-agarose beads. Precleared nuclear extract (10 μg) was incubated with PGE₂ (10 μmol/L), GW501516 (10 nmol/L), arachidonic acid (10 μmol/L), or vehicle DMSO (1:10,000 dilution) for 30 minutes at 4°C followed by overnight incubation with biotinylated DRE oligonucleotide at 4°C. The PPARδ-DRE complex was pulled down with streptavidin-agarose beads and subjected to Western blotting to detect bound PPARδ. Addition of arachidonic acid to the nuclear extract promotes PPARδ binding to DRE, whereas PGE₂ has no effect in the same system. The level of arachidonic acid-induced PPARδ binding is comparable with that induced by GW501516. The binding specificity was verified by the absence of binding in cold competitive control (biotinylated DRE oligonucleotide admixed with 100-fold of unlabeled DRE oligonucleotide) or with omission of cell lysate. *Bottom*, inhibition of COX has no effect on A23187-induced PPARδ binding to DRE in human HCC cells. Serum-starved HuH7 cells were incubated overnight with the COX inhibitors indomethacin (30 μmol/L) or NS-398 (30 μmol/L) or vehicle DMSO before incubation with A23187 (1 μmol/L, 20 minutes). At the end of treatment, the whole-cell lysates were obtained as described in Materials and Methods. Cell lysate (50 μg) was precleared and then incubated overnight with the biotinylated DRE. The PPARδ-DRE complex was pulled down and subjected to Western blotting for detection of bound PPARδ. Indomethacin and NS-398 had no influence on A23187-induced PPARδ binding to DRE. All the experiments were repeated thrice. **C**, inhibition of COX has no effect on cPLA₂α overexpression-induced DRE reporter activity. HuH7 cells were cotransfected with DRE and cPLA₂α expression plasmid or control vector pMT-2 for 3 hours followed by treatment with indomethacin (30 μmol/L) or vehicle DMSO overnight. Although overexpression of cPLA₂α enhances the DRE reporter activity (**, $P < 0.01$, compared with control vector), this effect is not inhibited by indomethacin ($P > 0.05$). *Columns*, mean of three separate experiments; *bars*, SD. **D**, the PGE₂-induced DRE reporter activity is blocked by the p38 MAPK inhibitor (SB203580) and the p42/44 MAPK inhibitor (PD98059) but not by the PI3K/Akt inhibitor (LY294002). HuH7 cells were transfected with DRE reporter plasmid for 3 hours followed by treatment with indomethacin (30 μmol/L) or PGE₂ (10 μmol/L) plus SB203580 (10 μmol/L), PD98059 (25 μmol/L), or LY294002 (20 μmol/L) overnight. *, $P < 0.05$; **, $P < 0.01$, compared with vehicle control; ***, $P < 0.01$, compared with PGE₂ treatment ($n = 3$).

Figure 6. Mechanisms for PPAR δ -mediated human HCC cell growth. PPAR δ promotes human HCC cell growth through induction of COX-2 expression and PGE $_2$ synthesis. The produced PGE $_2$ phosphorylates and activates cPLA $_2\alpha$, thus releasing arachidonic acid for further PPAR δ activation and PGE $_2$ synthesis via COX-2. This positive feed-forward loop between PPAR δ and PG pathways likely plays an important role in the regulation of human HCC cell growth.



A23187 also significantly increased the binding of PPAR δ to its response element and this effect was completely blocked by the selective cPLA $_2$ inhibitor AACOCF $_3$ (Fig. 4C). These observations further support the role of cPLA $_2\alpha$ in PPAR δ activation. The fact that AACOCF $_3$ also inhibited PPAR δ activation in cells without A23187 treatment (Fig. 4A and C) suggests the presence of endogenous cPLA $_2\alpha$ for PPAR δ activation.

The effect of cPLA $_2\alpha$ on PPAR δ binding to its response element was further confirmed by the biotinylated DRE oligonucleotide immunoprecipitation assay. Under this assay system, activation of cPLA $_2\alpha$ by A23187 enhanced the binding of PPAR δ to DRE and that two structurally unrelated cPLA $_2\alpha$ inhibitors, AACOCF $_3$ and the 1,2,4-trisubstituted pyrrolidine derivative, prevented PPAR δ -DRE binding (Fig. 4D). The specificity of the assay was confirmed by the complete elimination of binding with the unlabeled DRE oligonucleotides.

Given that PGE $_2$ can phosphorylate and activate cPLA $_2\alpha$ and that cPLA $_2\alpha$ is implicated in PPAR δ activity, we next examined the effect of PGE $_2$ on PPAR δ activation in HuH7 cells and evaluated the role of cPLA $_2$ in this process. Figure 5A shows that PGE $_2$ treatment significantly increased the PPAR δ transcription activity as determined by transient transfection and reporter activity assays, and this effect was completely blocked by the cPLA $_2$ inhibitor AACOCF $_3$. These observations further support the role of cPLA $_2$ in PGE $_2$ -induced PPAR δ activation in human HCC cells.

To further delineate the effect of arachidonic acid and PGE $_2$ on PPAR δ activation, a cell-free system was used, in which the nuclear extracts from HuH7 cells were incubated with PGE $_2$ or arachidonic acid in the presence of biotinylated DRE oligonucleotide to determine the binding of PPAR δ to DRE *in vitro*. Addition of arachidonic acid to the nuclear extract induced the binding of PPAR δ to DRE, which is comparable with the effect induced by the synthetic PPAR δ ligand GW501516 (Fig. 5B). In contrast, PGE $_2$ failed to induce PPAR δ binding when directly added to the nuclear extract (Fig. 5B). These findings suggest that PGE $_2$ lacks the ability to directly activate PPAR δ , although arachidonic acid itself can bind PPAR δ and alter PPAR δ transcription activity. The latter assertion is further supported by the observation that the COX-2 inhibitors indomethacin and NS-398 had no apparent influence on A23187-induced PPAR δ binding to DRE (Fig. 5B). Thus, given that PGE $_2$ activates PPAR δ only in intact cell, its effect is most likely mediated through cPLA $_2\alpha$ phosphorylation-induced arachidonic

acid release rather than direct PPAR δ binding. In agreement with this assertion, forced expression of cPLA $_2\alpha$ enhanced DRE reporter activity and this effect was not blocked by the COX inhibitor indomethacin (Fig. 5C), although indomethacin alone decreased DRE reporter activity (Fig. 5D). In addition, the PGE $_2$ -induced PPAR δ transcriptional activity was inhibited by the p44/42 MAPK inhibitor (PD98059) and the p38 MAPK (SB203580; Fig. 5D). These findings are consistent with the effect of these protein kinase inhibitors on PGE $_2$ -induced cPLA $_2\alpha$ phosphorylation (Fig. 3B) and further support the role of cPLA $_2\alpha$ in PGE $_2$ -induced PPAR δ activation. In contrast to the reported involvement of the PI3K/Akt pathway in PGE $_2$ -induced PPAR δ activation in colorectal adenoma cells (40), in our system blocking PI3K/Akt by LY294002 did not affect PGE $_2$ -induced PPAR δ activation in HCC cells. Taken together, our results suggest that the MAPK-mediated cPLA $_2\alpha$ phosphorylation is an important mechanism for PGE $_2$ -induced PPAR δ activation in HCC cells.

In summary, this study shows an important role of PPAR δ in human HCC cell growth. The most novel mechanistic aspect of this study is the identification of cPLA $_2\alpha$ -controlled arachidonic acid metabolism for endogenous PPAR δ activation in the nucleus. The importance of cPLA $_2\alpha$ in PPAR activation can be explained by its unique characteristic of nuclear localization mediated by its NH $_2$ -terminal Ca $^{2+}$ -dependent lipid binding domain (CaLB or C2 domain). Our findings provide the first evidence for the activation of PPAR δ by cPLA $_2\alpha$ in human HCC cells. The observations that PPAR δ enhances COX-2 gene expression and that the COX-2-derived PGE $_2$ further activates PPAR δ through phosphorylation of cPLA $_2\alpha$ depict a novel cross-talk between PPAR δ and PG signaling pathways that coordinately regulate HCC cell growth (Fig. 6). It is conceivable that disruption of this feed-forward loop may represent an important future therapeutic strategy for the chemoprevention and treatment of human HCC.

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Cross-talk between Peroxisome Proliferator-Activated Receptor δ and Cytosolic Phospholipase $A_2\alpha$ /Cyclooxygenase-2/Prostaglandin E_2 Signaling Pathways in Human Hepatocellular Carcinoma Cells

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