Peroxisome proliferator-activated receptor-\(\delta\) (PPAR\(\delta\)) is a nuclear receptor implicated in lipid oxidation and the pathogenesis of obesity and diabetes. This study was designed to examine the potential effect of PPAR\(\delta\) on human cholangiocarcinoma cell growth and its mechanism of actions. Overexpression of PPAR\(\delta\) or activation of PPAR\(\delta\) by its pharmacological ligand, GW501516, at low doses (0.5–50 nM) promoted the growth of three human cholangiocarcinoma cell lines (CCLP1, HuCCT1, and SG231). This effect was mediated by induction of cyclooxygenase-2 (COX-2) gene expression and production of prostaglandin E\(_2\) (PGE\(_2\)) that in turn transactivated epidermal growth factor receptor (EGFR) and Akt. In support of this observation, inhibition of COX-2, EGFR, and Akt prevented the PPAR\(\delta\)-induced cholangiocarcinoma cell growth. Furthermore, PPAR\(\delta\) activation or PGE\(_2\) induced the phosphorylation of cytosolic phospholipase A\(_2\) (cPLA\(_2\)+), a key enzyme that releases a substrate for PG production via COX-2. Overexpression or activation of cPLA\(_2\)+ enhanced PPAR\(\delta\)-mediated transcription (DRE) and increased AA-derived prostaglandin E\(_2\) (PGE\(_2\)) production and tumor growth, whereas antisense depletion of COX-2 attenuates growth (8, 9). Consistent with these findings, selective COX-2 inhibitors prevented tumor cell growth and invasion, in vitro and in nude mice (8, 9, 12–14), although their effect may be mediated through COX-2-dependent and -independent mechanisms. Transactivation of EGFR and Akt has recently been proposed as one of the important mechanisms for COX-2 and PGE\(_2\)-mediated cholangiocarcinoma cell growth (15).

COX, including COX-1 and COX-2, is the rate-limited enzyme catalyzing the conversion of arachidonic acid (AA) into endoperoxide intermediates that are ultimately converted by specific synthases to prostanoids, including PGE\(_2\), the most abundant PG in human cholangiocarcinoma cells (16–19). Whereas COX-1 is constitutively expressed in most cells, COX-2 is highly induced by inflammatory cytokines/chemokines, growth factors, oncone activation, and tumor promotion.

Cholangiocarcinoma is a highly malignant neoplasm of the biliary tree, accounting for about 10–15% of the primary liver cancers. It often arises from background conditions that cause long standing inflammation, injury, and reparative biliary epithelial cell proliferation, such as primary sclerosing cholangitis (PSC), clonorchiasis, hepatolithiasis, or complicated fibrocystic diseases (1–4). Although chronic inflammation and cellular injury within bile ducts contribute to partial obstruction of bile flow, apoptotic resistant cells and relevant predisposing factors in the pathogenesis of cholangiocarcinoma (1–4), the molecular mechanisms of inflammation and cholangiocarcinogenesis remain to be further defined.

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2 The abbreviations used are: COX-2, cyclooxygenase-2; AA, arachidonic acid; AAOCT\(_p\), arachidonoylthrombolipid ketone; cPLA\(_2\), cytosolic phospholipase A\(_2\)+; DRE, PPAR\(\delta\) response element; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; PG, prostaglandin; PGE\(_2\), prostaglandin E\(_2\); P3-kinase, phosphatidylinositol 3-kinase; PPAR\(\delta\), peroxisome proliferator-activated receptor-\(\delta\); siRNA, small interfering RNA; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; ELISA, enzyme-linked immunosorbent assay.
ers, thus contributing to the enhanced PG production when these signaling pathways are activated in inflammatory and neoplastic diseases (16–19). PGs transduce signals mainly through binding to their specific G protein-coupled receptors (GPCRs) along the plasma membrane. Although certain PGs including 15d-PGJ2 and PGI2 are known to activate peroxisome proliferators-activated receptors (PPARs) (20–22), the physiological implication of endogenous AA metabolism for PPAR activation in cells remains largely unknown.

PPARs belong to the nuclear hormone receptor superfamily and comprise of three subtypes: PPARα, PPARγ, and PPARδ/β. As ligand-activated transcription factors, they form heterodimers with the retinoid X receptor (RXR) and bind to their response elements (PPREs) in the promoters of target genes upon activation (23, 24). A large body of evidence has documented an important role of PPARs in various cellular functions and in the pathogenesis of several human diseases including diabetes, obesity, and hyperlipidemia. PPARα is highly expressed in hepatocytes and implicated in lipid catabolism (25–29), whereas PPARγ is predominantly expressed in adipose tissue and plays an important role in adipocyte differentiation, insulin sensitization, and glucose homeostasis (30–34). In contrast, PPARδ/β is ubiquitously expressed in most cells (26) and is implicated in fatty acid oxidation, cell differentiation, inflammation, cell motility, and cell growth (22, 35–44). More recently, emerging studies suggest a potential role of PPARδ in carcinogenesis. For example, the expression of PPARδ is elevated in human and rat colon carcinomas when compared with normal colon tissues (45). Exposure of Apc−/− mice to the PPARδ agonist, GW501516, increased the number of large adenomas and reduces the targeted homologous recombination (46). When the PPARδ−/−/Apc−/− cells were transplanted into Balb/c nude mice (48), these findings suggest a tumor suppressor role of PPARδ during intestinal carcinogenesis. Moreover, PPARδ has also been implicated in the growth of several human cancers, including hepatocellular carcinoma, breast cancer, and prostate cancer (49, 50). PPARδ is a downstream gene of Wnt-β-catenin signal pathway and the target of nonsteroidal anti-inflammatory drugs (NSAIDs), which are COX inhibitors with anti-tumor effect (45, 51). Moreover, PPARδ has also been shown to mediate the PGE2-induced intestinal adenoma growth (52). However, despite the documented tumor-promoting effect of PPARδ, there is also evidence suggesting that PPARδ might inhibit intestine tumor development (53). Therefore, the precise role of PPARδ in tumorigenesis remains to be further defined.

This study was designed to evaluate the effect and mechanisms of PPARδ in cholangiocarcinoma cell growth control. Our results demonstrate that overexpression of PPARδ or activation of PPARδ by its pharmacological ligand, GW501516, significantly enhances cholangiocarcinoma cell growth and this effect is mediated, at least in part, through induction of COX-2 expression and PGE2 production. Moreover, our data show that the COX-2-derived PGE2 further activates PPARδ through a novel cPLA2α-dependent mechanism, thus forming a positive feedback loop that coordinates promotes tumor cell growth.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture medium and Lipofectamine Plus™ reagent were purchased from Invitrogen. Cell proliferation reagent WST-1 was purchased from Roche Applied Science. [3H]Thymidine was from PerkinElmer Life Sciences. Luciferase Assay System and reporter lysis buffer were from Promega Corporation. Antibody providers are as follows: anti-COX-2 (Cayman Chemical Co.); anti-cPLA2α, anti-PPARδ, and anti-EGFR (Santa Cruz Biotechnology); anti-phospho-cPLA2α (Ser505) and Akt Kinase Assay kit (Cell Signaling Technology); anti-phospho-EGFR (BD Biosciences), and anti-β-actin (Sigma). Chemiluminescence detection reagent was from Amersham Biosciences. PPARδ agonist, GW501516, was purchased from Cayman Chemical Co. (Ann Arbor, MI). Prostaglandin E2, indomethacin, arachidonic acid, stearic acid, oleic acid, α-linolenic acid, A23187, the cPLA2 inhibitors AACOCF3 and pyrrolidine derivative (cat. 525143), the EGFR tyrosine kinase inhibitor AG1478, the p38 kinase inhibitor SB203580, the protein kinase C inhibitor bisindolylmaleimide I, the phosphatidylinositol 3-kinase inhibitor LY294002, and the p44/42 MAPK inhibitor PD98059 were purchased from Calbiochem. The PGE2 enzyme immunometric assay kit was purchased from Amersham Biosciences. PPARδ Knockdown Factor Assay kit and siRNA Control kit were from Cayman Chemical Co. (Ann Arbor, MI). The immobiulized streptavidin beads were purchased from Pierce. Poly(dI-dC) was from Amersham Biosciences.

**Cell Culture and WST-1 Assay**—Three cholangiocarcinoma cell lines, CCLP1, HuCCT1, and SG231 were cultured respectively in medium DMEM, RPMI 1640, and MEMa as previously described (8, 10, 15). 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 (3000/well) were seeded on 96-well plate and incubated at 37 °C overnight. The cells were then treated with GW501516 for indicated time periods. WST-1 (10 µl) was subsequently added to each well, and the culture continued for 30 min to 4 h prior to measurement of OD450 nm using an automatic enzyme-linked immunosorbent assay plate reader.

**[3H]Thymidine Incorporation**—The cells cultured in 24-well plates were incubated with different concentrations of GW501516 for 48 h. [3H]Thymidine (1 µCi/ml) was added to the medium during the last 4 h of culture. The cells were then washed twice with cold PBS and incubated with 5% trichloroacetic acid at 4 °C for 30 min to precipitate macromolecules. The precipitant was washed once with cold PBS and incubated with 2% SDS. The radioactivity was quantitated in a liquid scintillation counter.

**Transient Transfection and Luciferase Reporter Assay**—Cells were seeded in 6-well plate in culture medium containing 10% FBS the day before transfection. On the following day, the cells in each well (80–90% confluence) were transfected with 1 µg of
A Positive Feedback Loop between PPARδ and PGE₂ Pathways

plasmid using Lipofectamine Plus reagent (Plus reagent 6 µl, Lipofectamine 4 µl) in serum-free medium. For co-transfection with two plasmids, double volume of Lipofectamine Plus reagent was used. After 4 h of transfection, the transfection medium was replaced with culture medium containing 10% fetal bovine serum. After 16 h of incubation, the cells were washed three times in ice-cold PBS and lysed by reporter lysis buffer on ice for 20 min. The cells were then scraped down and spun at 14,000 rpm for 10 min in cold room. The supernatant was collected for luciferase activity assay using a Berthold AutoLumat LB 953 luminometer (Nashua, NH).

Preparation of Whole Cell Lysate and Immunoblotting—
CCLP1 and HuCCT1 cells were grown on 6-well plates and treated with different concentration of GW501516 for different time in 0.5% fetal bovine serum medium. The vehicle, Me₂SO, was added to the control culture. Following treatment for indicated time periods, 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 mM Hepes (pH 7.55), 1 mM EDTA, 1 mM dithiothreitol, and 1 mM mammalian protease inhibitor mixture (Sigma). The cell suspension
was placed on ice and sonicated for 15 s × 4. The samples were then centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatants were collected as whole cell lysate. The total protein concentration was measured by BCA reagent (Pierce). The cell lysate was aliquoted and frozen at −80 °C until use. For immunoblotting, 30 μg of protein was separated on 4–20% Tris-glycine gels and the separated proteins were electrophoretically transferred onto the nitrocellulose membrane (Bio-Rad). Nonspecific binding was blocked with 5% nonfat milk dissolved in buffer PBS-T (0.5% Tween 20 in buffer PBS) for 1 h at room temperature. The membrane was then incubated overnight with primary antibodies (1:1000 dilution for COX-2, EGFR, p-EGFR, Akt, p-Akt, and β-actin; 1:2000 dilution for PPARδ) in 5% milk PBS-T. Following repeated washing with PBS-T the next day, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) for 1 h at room temperature. After washing the blots were developed using the ECL Western blotting detection system and exposed to Eastman Kodak MR radiographic films.

**Immunoprecipitation and Western Blotting for cPLA₂α Phosphorylation**—To immunoprecipitate cPLA₂α, 500 μl of whole CCLP1 cell lysate (about 40 μg protein) in a 1.5-ml Eppendorf tube was precleared with 20 μl of protein A/G-agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. The cleared cell lysate was then incubated with 5 μl of mouse anti-human cPLA₂α monoclonal antibody at 4 °C for 3 h, with gentle agitation. 20 μl of protein A/G-agarose was then

![Figure 1](http://www.jbc.org/)

**D**

**a** CCLP1

**b** HuCCT1

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**E**

![Relative Luc Activity](http://www.jbc.org/)

**Figure 1—continued**
A Positive Feedback Loop between PPARδ and PGE₂ Pathways

FIGURE 2. PPARδ enhances COX-2 expression and PGE₂ production.

A. Time course of COX-2 protein level expression in CCLP1 cells. CCLP1 cells (80–90% confluence) were treated with 30 μM GW501516 (0.5–100 nM) for indicated time points. (The in vitro experiment was repeated three times. The cell lysate was collected. 30 μg of protein was added, and the sample was kept at 4 °C for 16 h, with gentle agitation, to precipitate cPLA₂-body complex. The protein A/G-agarose pellet was collected by centrifugation and washed four times with cold homogenization buffer at 4 °C. 20 μl of SDS sample loading buffer was then added to the pellet, and the mixture was boiled for 5 min prior to SDS-PAGE. After centrifugation, the supernatant was collected and centrifuged to precipitate cPLA₂. The cleared nuclear extract from treated cells or control cells were then added to the dsDNA-coated well and incubated at 4 °C overnight. After complete washing, PPARδ overexpression on COX-2 protein level was measured by the protein concentration detected by BCA reagent.)

B. Effect of GW501516 on PGE₂ production in HuCCT1 cells. HuCCT1 cells were treated with GW501516 (0.5–100 nM) for indicated time points. (Each experiment was repeated three times. The supernatant was collected and centrifuged to determine PGE₂ production. The values are expressed as mean ± S.D. (*, p < 0.05).)

C. Effect of GW501516 on PGE₂ production in H9262 cells. H9262 cells (80–90% confluence) were serum-starved for 24 h before treatment with GW501516 (0.5–100 nM) for indicated time points. (The in vitro experiments were performed three times. The samples were subjected to SDSPAGE and Western blot analysis to determine COX-2 protein level. Each experiment was repeated three times. The values are expressed as mean ± S.D. (*, p < 0.05).)

D. Effect of GW501516 on PGE₂ production in H9262 cells. H9262 cells (80–90% confluence) were serum-starved for 24 h before treatment with GW501516 (0.5–100 nM) for indicated time points. (The in vitro experiments were performed three times. The samples were subjected to SDS-PAGE and Western blot analysis to determine COX-2 protein level. Each experiment was repeated three times. The values are expressed as mean ± S.D. (*, p < 0.05).)

### Retracted Section

**Purification of Nuclear Extract**—CCLP1 cells cultured in serum-free medium in 6-well plates were treated as indicated in the text. The supernatant was collected and centrifuged to remove floating cells. 100 μl of each sample was used to measure PGE₂ level using the PGE₂ enzyme immunoassay system as previously described (54, 55).

**Measurement of PGE₂ Production**—CCLP1 cells cultured in 100-mm dishes at 80–90% 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 min on ice. Following homogenization using 27-gauge sterile needle on ice, the nuclei were pelleted by centrifugation at 600 × g for 10 min. The nuclei were then washed three times in the isotonic buffer and resuspended in HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 1 mM dithiothreitol, and 0.5% of Nonidet P-40) 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 min at 4 °C. The supernatant was then used as nuclear extract and frozen at −80 °C until use. Aliquots of nuclear extracts were used to precipitate the protein concentration detected by BCA reagent.

**Biotinylated DRE Oligonucleotide Precipitation Assay**—The assay was performed as previous reported with modification (56). The nucleotide sequences of biotinylated PPARδ response element (DRE) were 5'-GCGTGAGCGCTACAGTGTCATATTCG-3' and 5'-CCGATTGACCTGTGAGCCGC-3' (45). These two complementary strands were annealed from Cayman (Ann Arbor, MI). Briefly, the oligonucleotide containing the PPARδ binding consensus sequence was immobilized onto the bottom of wells. 50 μg of nuclear extract from treated cells or control cells were added to the dsDNA-coated well and incubated at 4 °C overnight. After complete washing, PPARδ antibody was added, and the samples were incubated at room temperature for 1 h. The HRP-conjugated secondary antibody and developing solution were sequentially added and the OD₆₅₅ nm value was determined.

The biotinylated DRE oligonucleotide precipitated antibody was added to the dsDNA-coated well and incubated at 4 °C overnight. After complete washing, PPARδ antibody was added, and the samples were incubated at room temperature for 1 h. The HRP-conjugated secondary antibody and developing solution were sequentially added and the OD₆₅₅ nm value was determined.
**A Positive Feedback Loop between PPARδ and PGE2 Pathways**

**RESULTS**

**PPARδ Promotes Cholangiocarcinoma Cell Growth**—The effect of PPARδ on human cholangiocarcinoma cell growth was evaluated by PPARδ overexpression or treatment with GW501516, a selective PPARδ ligand. As shown in Fig. 1A, GW501516 treatment significantly increased the growth of three human cholangiocarcinoma cell lines (CCLP1, HuCCT1, and SG231), as determined by the WST-1 assay. This effect was dose-dependent (0.5-50 nM) and was observed at different treatment periods (24–72 h). The dose-dependent effect of

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10 μg of poly(dI-dC)-poly(dI-dC) for 16 h. DRE-bound protein was pulled down by incubating the samples with 25 μl of streptavidin–agarose beads for 1 h 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, and the samples were subjected to SDS-PAGE and Western blotting to detect PPARδ.

**Fatty Acid-Protein Overlay Assay**—This assay was performed as previous report with modification briefly, various amounts of fatty acid were spotted to nitrocellulose membrane (American Blotting Sci.) and completely dried. The blot was re-wet in deionized water and then blocked in 3% fatty acid-free bovine serum albumin (FAF-BSA)/PBS-T (0.05% Tween 20) for 1 h at room temperature. The blot was then incubated overnight with 0.24 μg/ml human recombinant PPARδ (Cayman Chemical) in 1.5% FAF-BSA/PBS-T at 4 °C. The blot was washed gently and incubated with anti-PPARδ antibody (1:1000) for 1 h followed by incubation with secondary antibody for additional 1 h at room temperature and developed using ECL.

**Statistical Analysis**—Statistical analysis was performed using Microsoft Excel 2003 software. Comparisons were performed using Student’s t test. Values of \( p < 0.05 \) were considered statistically significant.
GW501516 on cell growth was also confirmed by the [3H]thymidine incorporation assay (Fig. 1B). The PPARδ protein level was similar among the three cholangiocarcinoma cell lines utilized in this study (Fig. 1C). Consistent with the effect of GW501516, overexpression of PPARδ also significantly increased the growth of human cholangiocarcinoma cells, as determined by both WST-1 and [3H]thymidine incorporation assays (Fig. 1D). The transcriptional activity of PPARδ in these cells was verified by determining the reporter activity of a luciferase promoter construct containing the PPARδ response element (DRE) (45). As shown in Fig. 1E, treatment of the PPARδ ligand, GW501516, significantly increased the DRE-driven luciferase reporter activity (~2-fold, p < 0.01). Overexpression of PPARδ alone or in combination with GW501516 further enhanced the DRE reporter activity (5.8 and 6.9-fold, respectively, p < 0.01). These observations reveal a growth-stimulatory effect of PPARδ in human cholangiocarcinoma cells.

**PPARδ Activation Induces COX-2 Expression and PGE2 Production**—Further experiments were performed to determine the mechanisms by which PPARδ promotes human cholangiocarcinoma growth. Because COX-2-derived PGE2 has been implicated in cholangiocarcinogenesis, we reasoned...
COX-2 and PGE₂ signaling might play a role in PPARδ-induced cholangiocarcinoma cell growth. Indeed, an increased PGE₂ production in human cholangiocarcinoma cells induced by PPARδ overexpression and GW501516 treatment. Furthermore, the EGFR tyrosine kinase inhibitor, AG1478, and the PI 3-kinase inhibitor, LY294002, both blocked the PPARδ overexpression or GW501516-induced cell growth (Fig. 4, B and D). These observations suggest the involvement of COX-2, EGFR, and Akt signaling in PPARδ-mediated cholangiocarcinoma cell growth.

**PPARδ Induces cPLA₂α Phosphorylation through COX-2-mediated PGE₂ Production—cPLA₂α is the rate-limiting enzyme that releases arachidonic acid from membrane phospholipids and thus provides a substrate for COX enzymes.** The EGFR and COX-2 controlled PG synthesis has been implicated in cholangiocarcinoma cell growth (4). To determine whether PPARδ can further activate the EGFR-Akt signaling pathway, we sought to further determine whether PPARδ-induced PGE₂ plays an important role in PPARδ-induced cholangiocarcinoma cell growth (4).

**A Positive Feedback Loop between PPARδ and PGE₂ Pathways**

COX-2 and PGE₂ signaling might play a role in PPARδ-induced cholangiocarcinoma cell growth. Indeed, an increased PGE₂ production in human cholangiocarcinoma cells induced by GW501516 significantly increased the expression of COX-2, EGFR, and Akt signaling in PPARδ-induced Cholangiocarcinoma Cell Growth (4).

**Involvement of COX-2/PGE₂-mediated Transactivation of EGFR and Akt in PPARδ-induced Cholangiocarcinoma Cell Growth**—Given that COX-2-derived PGE₂ has been shown to promote cholangiocarcinoma cell growth through activation of EGFR and Akt (15), we next determined the potential effect of PPARδ on EGFR and Akt phosphorylation. As shown in Fig. 3A, treatment of CCLP1 cells with COX-2 expression plasmid enhanced the phosphorylation of both Akt and EGFR, whereas the levels of total Akt and EGFR were not altered. The GW501516-induced phosphorylation of Akt and EGFR was blocked by siRNA inhibition of COX-2 (Fig. 3B). Furthermore, overexpression of PPARδ in CCLP1 cells also increased the phosphorylation of EGFR/Akt, and this effect was blocked by siRNA inhibition of COX-2 (Fig. 3C). These findings suggest that PPARδ activates EGFR and Akt in human cholangiocarcinoma cells, and this effect is mediated, at least in part, through COX-2.

The role of COX-2/EGFR/Akt signaling in PPARδ-induced cholangiocarcinoma cell growth was further documented. As shown in Fig. 4, A and C, siRNA inhibition of COX-2 prevented the growth of CCLP1 and HuCCT1 cells induced by PPARδ overexpression and GW501516 treatment. Furthermore, the EGFR tyrosine kinase inhibitor, AG1478, and the PI 3-kinase inhibitor, LY294002, both blocked the PPARδ overexpression or GW501516-induced cell growth (Fig. 4, B and D). These observations suggest the involvement of COX-2, EGFR, and Akt signaling in PPARδ-mediated cholangiocarcinoma cell growth.

**cPLA₂α Enhances DRE Reporter Activity**—Although recent evidence suggests the involvement of cPLA₂α in the activation of PPARα and PPARγ in primary and transformed hepatocytes and lung epithelial cells (62, 63), the potential role of cPLA₂α in PPARδ activation has not been investigated. In this study, the direct effect of cPLA₂α on PPARδ activation was investigated in human cholangiocarcinoma cells. For these experiments, CCLP1 cells were cotransfected with cPLA₂α expression plasmid or control vector pMT-2 and DRE luciferase reporter construct. As shown in Fig. 7A, overexpression of cPLA₂α significantly increased the expression of COX-2, EGFR, and Akt signaling in PPARδ-induced cholangiocarcinoma cell growth. Indeed, activation of PPARδ and COX-2 phosphorylation of Akt and EGFR was blocked by siRNA inhibition of COX-2 (Fig. 3). Furthermore, overexpression of PPARδ increased the phosphorylation of both EGFR and Akt, whereas the levels of total Akt and EGFR were not altered. The GW501516-induced phosphorylation of Akt and EGFR was blocked by siRNA inhibition of COX-2 (Fig. 3B). Furthermore, overexpression of PPARδ in CCLP1 cells also increased the phosphorylation of EGFR/Akt, and this effect was blocked by siRNA inhibition of COX-2 (Fig. 3C). These findings suggest that PPARδ activates EGFR and Akt in human cholangiocarcinoma cells, and this effect is mediated, at least in part, through COX-2.

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**A Positive Feedback Loop between PPARδ and PGE2 Pathways**

**FIGURE 6.** PPARδ activates cPLAα via PGE2. A, activation of PPARδ by GW501516 induces cPLAα phosphorylation. CCLP1 cells at 80% confluence were serum-starved overnight and then treated with GW501516 (10 nM) for indicated time periods. The cell lysates were collected and subjected to immunoprecipitation and Western blot analysis to determine cPLAα phosphorylation. B, overexpression of PPARδ induces cPLAα phosphorylation. CCLP1 cells at 80% confluence were transfected with the PPARδ expression plasmid or the control vector (SGS) (暴露 to Lipofectamine Plus reagent, 10 nM) for 4 h. After transfection, the cells were incubated in serum-free medium for 24 h. The whole cell lysate was subjected to immunoprecipitation and Western blot analysis to determine cPLAα phosphorylation. C, COX-2 prevents GW501516-induced cPLAα phosphorylation. CCLP1 cells were transfected with COX-2 siRNA or non-target control siRNA for 4 h using Lipofectamine 2000 reagent. The transfection medium was then replaced by serum-free DMEM containing 10 nM of GW501516 or vehicle and the cells were incubated overnight. On the following day, the cells were washed with cold PBS and the cell lysate was obtained for luciferase activity assay to determine the DRE reporter activity. The values represent mean ± S.D. from three experiments (*, p < 0.01 compared with vector). The level of cPLAα in these cells was determined by Western blot analysis. B, activation of cPLAα by COX-2 prevents GW501516-induced cPLAα phosphorylation. CCLP1 cells transfected with COX-2 siRNA or non-target control siRNA for 4 h using Lipofectamine 2000 reagent. The transfection medium was then replaced by serum-free DMEM containing 10 nM of GW501516 or vehicle and the cells were incubated overnight. On the following day, the cell lysate was collected and subjected to luciferase activity assay to determine the DRE reporter activity. The values represent mean ± S.D. from three experiments (*, p < 0.01 compared with vector).

**FIGURE 7.** cPLAα enhances PPARδ reporter activity in CCLP1 cells. A, cPLAα overexpression enhances PPARδ reporter activity. CCLP1 cells at 80% confluence were cotransfected with the human cPLAα expression plasmid or the control vector pMT2 plus the DRE reporter construct for 4 h using Lipofectamine Plus reagent. The transfection medium was then replaced by fresh serum-free DMEM and the cells were incubated overnight. On the following day, the cells were washed with cold PBS and the cell lysate was obtained for luciferase activity assay to determine the DRE reporter activity. The values represent mean ± S.D. from three experiments (*, p < 0.01 compared with vector). The level of cPLAα in these cells was determined by Western blot analysis. B, activation of cPLAα by the calcium ionophore A23187 enhances DRE reporter activity. CCLP1 cells transfected with DRE reporter gene were incubated in serum-free medium overnight. On the following day, A23187 (10 μM), the cPLAα inhibitor (AACOCF3, 20 μM) or vehicle was added to the cells and the culture was continued for 4 h. The cell lysate was then collected to determine the luciferase reporter activity. The values represent mean ± S.D. from three experiments (*, p < 0.01 compared with vehicle).

Consistently increased the DRE reporter activity (3.2-fold of control, p < 0.01). Consistent with this, activation of cPLAα by the calcium ionophore A23187 (1 μM) also significantly increased the PPARδ transcription activity in CCLP1 cells (4.5-fold of control, p < 0.01), which was inhibited by the cPLAα inhibitor AACOCF3 (20 μM) (Fig. 7B). These findings demonstrate a direct role of cPLAα for PPARδ activation.

**cPLAα Enhances PPARδ Binding to DRE in CCLP1 Cells**—The role of cPLAα in PPARδ activation was further examined by assessing the binding of PPARδ to DRE, in vitro. For this purpose, two complementary approaches were utilized, 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. 8A, overexpression of cPLAα or activation of cPLAα by the calcium ionophore, A23187, significantly increased the binding of PPARδ to its response element, as determined by the ELISA-based nuclear transcription factor assay. The effect of cPLAα transfection or A23187 treatment appeared slightly less than that induced by the PPARδ ligand, GW501516. Furthermore, selective cPLAα inhibition with the selective cPLAα inhibitor AACOCF3 (20 μM) (Fig. 8A) suggests that cPLAα on PPARδ binding to DRE was also mediated by the biotinylated DRE oligonucleotide immobilization assay. Under this assay system, transfection of cPLAα expression plasmid in CCLP1 cells also increased the
binding of PPARδ to DRE (Fig. 8B). The specificity of the assay was confirmed by the complete elimination of binding with the unlabeled DRE oligonucleotides. Similarly, the data from the biotinylated DRE oligonucleotide precipitation assay also confirmed that activation of cPLA2α by A23187 enhanced the binding of PPARδ to DRE and that two structurally unrelated cPLA2α inhibitors, AAOCCF3 and the pyrrolidine derivative, prevented PPARδ-DRE binding (Fig. 8, C–E).

**cPLA2α Induces COX-2 Gene Expression**—The results presented in the above sections indicate that cPLA2α-mediated AA metabolites can activate PPARδ. This finding, along with the observation that PPARδ activation increases COX-2 expression, prompted us to evaluate the effect of cPLA2α on COX-2 gene expression. For this approach, the CCLP1 cells transfected with the cPLA2α expression plasmid or control vector were cotransfected with a luciferase reporter construct under the control of the COX-2 gene promoter, and the cell lysates were obtained to determine luciferase reporter activity. As shown in Fig. 9, overexpression of cPLA2α significantly increased COX-2 gene transcription activity (4.5-fold of control, p < 0.01) as well as COX-2 protein level and PGE2 production (0.34 versus 0.18 ng/ml, p < 0.05). These findings are consistent with the activation of PPARδ by cPLA2α and the induction of COX-2 expression by PPARδ, as described in the above sections.

**PGE2 Activates PPARδ through cPLA2α in CCLP1 Cells**—Given that PGE2 can phosphorylate and activate cPLA2α, we predicted that cPLA2α is implicated in PPARδ activation. The treatment of human cholangiocarcinoma cells should also induce PPARδ activity. To measure the effect of PGE2 on PPARδ in CCLP1 cells, indeed, we significantly increased PPARδ activity (versus controls; this effect was blocked by inhibition of cPLA2α (LY294002), the p44/42 MAPK inhibitor (SB203580), the p38 MAPK inhibitor (SB203580), and the Akt inhibitor (AAOCCF3). These data support the role of PGE2-induced cPLA2α phosphorylation for PPARδ activation in human cholangiocarcinoma cells.

To further delineate the effect of AA and PGE2 on PPARδ activation, an in vitro system was employed, in which recombinant human PPARδ was incubated with different fatty acids or PGE2 in the presence of biotinylated DRE oligonucleotide to determine the effect of fatty acids and PGE2 on PPARδ-DRE binding. As shown in Fig. 10A, addition of 500 nM AA induced the binding of PPARδ to DRE. In contrast, three other fatty acids, including α-linolenic acid, oleic acid, and stearic acid, failed to induce PPARδ binding. PGE2 also failed to induce PPARδ binding to DRE under similar conditions (up to 10 μM). Consistent with these results, fatty acid-protein overlay assay showed that AA directly bound PPARδ in a dose-dependent manner (Fig. 10C). These findings suggest that PGE2 lacks the ability to directly activate PPARδ, although AA itself can bind PPARδ and alter PPARδ transcription activity. The latter assertion is further supported by the observation that the COX-2 inhibitor, indomethacin, had no apparent influence on A23187-induced PPARδ binding to DRE (Fig. 10D). Thus, given that PGE2 activates PPARδ only in intact cells, its effect is most likely mediated through cPLA2α phosphorylation-induced AA release rather than direct PPARδ binding.

**DISCUSSION**

This study reveals an important role of PPARδ in human cholangiocarcinoma cell growth. Our data show that PPARδ overexpression or activation enhances human cholangiocarcinoma cell growth and this effect is mediated, at least in part, through induction of COX-2 gene expression and PGE2 synthesis. Moreover, the COX-2-derived PGE2 further activates PPARδ through phosphorylation of cPLA2α. The interactions between PPARδ and PG signaling pathways form a positive feedback loop that likely plays an important role in cholangiocarcinoma cell growth (Fig. 11). The most novel mechanistic aspect of this study is, perhaps, the identification of cPLA2α-controlled AA metabolism for endogenous PPARδ activation.

Activation of PPARδ involves ligand-induced conformational change which alters the binding of PPAR with other nuclear proteins and the basal transcriptional machinery. Although AA metabolites represent several ligands for PPAR activation, the individual role of PPARδ in the control of eicosanoid production remains to be further defined. This report provides evidence for the activation of PPARδ by AA, which includes: 1) cPLA2α reporter activity in CCLP1 cells; 2) activation of the ionophore A23187; and 3) PPARδ reporter activity in CCLP1 cells with a specific cPLA2 inhibitor, AAOCCF3.

In addition to the data from the PPARδ reporter assay, our results also demonstrate that AA transactivation or activation enhanced the association of PPARδ to its specific DNA response element and this binding was blocked by inhibition of cPLA2α. Thus, cPLA2α activity is involved in PPARδ trans-activation, which underscores the importance of cPLA2α in PPARδ-mediated gene transcription.

The importance of cPLA2α in PPARδ activation can be explained by its unique characteristic of nuclear localization. The cPLA2α protein translocates from cytoplasm to nuclear envelope (7, 38–41) in response to calcium influx and this effect is mediated by its N-terminal Ca2+-dependent lipid binding domain (CaLB or C2 domain) (42, 64). As cPLA2α protein requires Ca2+ for its nuclear translocation, calcium ionophore A23187 was used in this study for maximal enzyme activation. Our data indicate that ionophore A23187 increased PPARδ reporter activity and DNA binding in CCLP1 cells, which was blocked by the cPLA2 inhibitor, AAOCCF3. These observations further support the involvement of calcium-mediated cPLA2α translocation in PPARδ-mediated gene transcription.

In this study, the role of PPARδ in cholangiocarcinoma growth was documented by utilization of the pharmacological PPARδ ligand (GW501516) and PPARδ overexpression. GW501516 is a synthetic pharmacological ligand that is selective for PPARδ with no effect on PPARα or PPARγ (even at dose as high as 10 μM) (35, 47, 64). In our system, we found that GW501516 was able to induce PPARδ activation and enhance cholangiocarcinoma cell growth at relatively low doses (0.5–50 nM). These findings are consistent with the observations that GW501516 enhances the growth of...
A Positive Feedback Loop between PPARδ and PGE₂ Pathways

A

![Graph showing relative binding activity with various treatments.]

- **GW501516 (5nM)**
- **cPLA₂α**
- **A23187**
- **AACOCF₂**

B

![Image showing protein expression with different treatments.]

C

![Image showing PPARδ protein expression with different treatments.]

D

![Image showing time course of PPARδ protein expression.]

E

![Image showing effects of Pyrrolidine and A23187 on PPARδ expression.]

Retracted October 19, 2015
Our data indicate that overexpression or activation of PPARγ induces COX-2 expression in human cholangiocarcinoma cells. The role of COX-2 in PPARγ-mediated cholangiocarcinoma cell growth was supported by our observation that siRNA inhibition of COX-2 prevented PPARγ-induced cell growth. The exact mechanism for PPARγ-induced COX-2 expression, however, remains to be further defined. Because homologous sequence analysis revealed no DRE site in the human COX-2 gene promoter, the possibility of PPARγ effect independent of DRE-binding cannot be excluded, giving that PPARα and PPARγ are known to mediate their effect through interaction with other transcription factors (21, 65, 66), in addition to binding PPRE.

PGE2 has been shown to promote tumor cell growth through mechanisms including cell proliferation, anti-apoptosis, invasion, and angiogenesis. Certain prostanoids, including PGE2, have been shown to feed-forwardly increase COX-2 expression and PGE2 production.

**FIGURE 8.** cPLA2α enhances PPARγ binding to its response element in CCLP1 cells. A, effect of cPLA2α overexpression, A23187, and AACCOCF3 on PPARγ binding (ELISA-based PPARγ transcription factor assay). Nuclear extracts (50 μg each sample) from CCLP1 cells treated with GW501516 (5 nM) for 4 h, A23187 (1 μM) alone or in combination with the cPLA2α inhibitor (AACCOCF3, 20 μM) for 4 h, or transfected with the cPLA2α expression plasmid, were added into PPARγ response element-coated 96-well plate and incubated overnight to allow protein-DNA binding. After complete wash, anti-PPARγ antibody was added to sample wells and incubated for 1 h. Subsequently, HRP-conjugated secondary antibody and color development solution were added and OD455 nm was measured (*, p < 0.05 compared with control; **, p < 0.01 compared with control). B, overexpression of cPLA2α enhances PPARγ binding to DRE (biotinylated DRE oligonucleotide precipitation assay). Whole cell lysate from CCLP1 cells transfected with the cPLA2α expression plasmid or the control vector pMT2 was precleared and then incubated with biotinylated DRE (1 μg) alone or with cold DRE oligonucleotide (no biotin modulation) (10 μg) overnight to allow PPARγ binding to DRE. Immobilized streptavidin-agarose beads were then added to pull-down the protein-DNA complex for Western blot detection of bound PPARγ as described under “Experimental Procedures.” C, A23187 treatment enhances PPARγ binding to DRE (biotinylated DRE oligonucleotide precipitation assay). This effect was blocked by pretreatment with the cPLA2α inhibitor, AACCOCF3. Whole cell lysates from CCLP1 cells treated with A23187 (1 μM, 20 min) in the presence or absence of AACCOCF3 (30 μM, 4 h) were precleared and then incubated overnight with biotinylated DRE (1 μg) in the presence or absence of cold DRE oligonucleotide (10 μg). Immobilized streptavidin-agarose beads were then added, and the samples were processed for biotinylated DRE oligonucleotide precipitation assay to detect bound PPARγ. D, cPLA2α inhibitor, AACCOCF3, prevents PPARγ binding to DRE in CCLP1 cells under basal culture conditions (biotinylated DRE oligonucleotide precipitation assay). Whole cell lysates from CCLP1 cells treated with AACCOCF3 (30 μM) at indicated time points were precleared and then incubated overnight with 1 μg of biotinylated DRE in the presence or absence of 10 μg of cold DRE oligonucleotide. Immobilized streptavidin-agarose beads were then added, and the samples were processed for biotinylated DRE oligonucleotide precipitation assay to detect bound PPARγ. E, effect of a separate structurally unrelated cPLA2α inhibitor, pyrrolidine derivative, on PPARγ binding to DRE. Whole cell lysates from CCLP1 cells treated with pyrrolidine derivative (2 μM) alone or in combination with A23187 (1 μM) at indicated time points were precleared and then incubated overnight with 1 μg of biotinylated DRE in the presence or absence of 10 μg of cold DRE oligonucleotide. Following addition of streptavidin-agarose beads, the samples were processed for biotinylated DRE oligonucleotide precipitation assay to detect bound PPARγ. All the experiments were repeated three times.
**FIGURE 10.** PGE$_2$ activates PPAR$_\delta$ through cPLA$_2$ in CCLP1 cells. **A**, PGE$_2$ treatment increases DRE reporter activity; this effect is blocked by inhibition of cPLA$_2$ activation. CCLP1 cells (80% confluence in 6-well plate) were transfected with human DRE reporter gene (1 $\mu$g) for 4 h using Lipofectamine Plus reagent. The transfection solution was then replaced by serum-free medium, and the cells were incubated overnight. On the following day, the cells were pre-incubated with the inhibitors of PI3-K (LY294002, 20 $\mu$M), p44/42 (PD98059, 20 $\mu$M), p38 (SB203580, 10 $\mu$M) or cPLA$_2$ (AACOCF$_3$, 20 $\mu$M) for 30 min, followed by PGE$_2$ treatment (10 $\mu$M) for additional 30 min. The cell lysates were obtained for luciferase activity assay to determine DRE reporter activity. The values are expressed as mean $\pm$ S.D. from three experiments (*, $p < 0.05$ compared with control; **, $p < 0.01$ compared with PGE$_2$ treatment).

**B**, AA induces PPAR$_\delta$ binding to its response element DRE. Human recombinant PPAR$_\delta$ (hrPPAR$_\delta$) (0.12 $\mu$g) was incubated with AA (500 nM), stearic acid (500 nM), oleic acid (500 nM), $\alpha$-linolenic acid (500 nM), PGE$_2$ (10 $\mu$M), or vehicle in Buffer A for 20 min. 0.2 $\mu$g of biotin-labeled DRE-streptavidin beads (with or without 10-fold cold competitive DRE) were then added, and the samples were incubated for an additional 20 min. After washing four times with buffer A, SDS sample buffer was added to the pellet, and the samples were subjected to SDS-PAGE and Western blotting to detect PPAR$_\delta$.

**C**, AA directly binds PPAR$_\delta$ in vitro (fatty acid-protein overlay assay). Different amounts of AA in 5 $\mu$L of volume were spotted onto the Hybond-C membrane. The blot was dried, re-wet, and blocked in 3% fatty acid-free bovine serum albumin (FAF-BSA)/PBS-T (0.05% Tween 20). The blot was then incubated with 0.24 $\mu$g/ml human recombinant PPAR$_\delta$, followed by sequential incubation with anti-PPAR$_\delta$ antibody (1:1000) and second antibody prior to development using ECL.

**D**, inhibition of COX by indomethacin had no effect on A23187-induced PPAR$_\delta$ binding to DRE in CCLP1 cells. Serum-starved CCLP1 cells were incubated with indomethacin (30 $\mu$M, overnight) prior to A23187 treatment (1 $\mu$M, 20 min). The whole cell lysates were obtained and incubated overnight with biotinylated DRE oligonucleotide (1 $\mu$g) (with or without 10 $\mu$g of cold competitive DRE). Streptavidin-agarose beads were then added to pull-down the protein-DNA complex for PPAR$_\delta$ Western blot. All the experiments were repeated three times.
Taken together, these studies reveal a novel cross-talk between PPARδ and PG signaling pathways that coordinate to regulate cholangiocarcinoma cell growth. Thus, interruption of this feed-forward loop may provide a novel therapeutic strategy for future chemoprevention and treatment.

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Mechanisms of Signal Transduction: A Novel Positive Feedback Loop between Peroxisome Proliferator-activated Receptor-δ and Prostaglandin E₂ Signaling Pathways for Human Cholangiocarcinoma Cell Growth

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