Featured Article

Up-regulation of p21 Gene Expression by Peroxisome Proliferator-Activated Receptor γ in Human Lung Carcinoma Cells

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Abstract

Purpose: The peroxisome proliferator-activated receptor γ (PPARγ), a ligand-dependent transcription factor belonging to the family of nuclear receptors, has been implicated in the regulation of cell growth and differentiation although the exact mechanism(s) of this activity has not been elucidated. In this study, we explored the role of PPARγ signaling on the control of gene expression of the cell cycle-dependent kinase inhibitor p21 in human lung carcinoma cells.

Experimental Design: Using several human lung carcinoma cell lines (small and non-small carcinoma cells), we assayed for cell growth inhibition and apoptosis induction. We also assayed for p21 mRNA and protein expression by reverse transcription-PCR, real-time reverse transcription-PCR, and Western blot analysis. Nuclear protein binding activities to three response elements located in the p21 promoter [nuclear factor (NF)-κB, Sp1, and NF-interleukin 6 (IL6) CAAT/enhancer binding protein (C/EBP)] were measured by gel mobility shift assays. We used transient transfection assays with p21 promoter reporter gene constructs to determine the transcriptional regulation by PPARγ ligands. Finally, by using p21 antisense oligonucleotides, we tested the link between PPARγ activation and p21 signaling in cell growth inhibition assays and by Western blot analysis.

Results: We showed that the PPARγ ligands PGJ2 and ciglitazone inhibit the growth and induce the apoptosis of several human lung carcinoma cell lines, whereas the PPARα agonist WY14643 has little effect. Treatment of lung carcinoma cells with the PPARγ ligands PGJ2, ciglitazone, troglitazone, and GW1929 elevated p21 mRNA and protein levels and reduced cyclin D1 mRNA levels. These results were supported by transient transfection assays, which indicated that PPARγ ligands increased p21 gene promoter activity in human lung carcinoma cells. In addition, p21 antisense oligonucleotides inhibited PPARγ ligand-induced p21 protein expression and significantly blocked lung carcinoma cell growth inhibition induced by PPARγ ligands. Finally, electrophoresis mobility shift experiments demonstrated that PPARγ ligands increased the nuclear binding activities of Sp1 and NF-IL6 (C/EBP), two transcription factors with regulatory elements in the promoter region of the p21 gene.

Conclusion: PPARγ ligands inhibit human lung carcinoma cell growth and induce apoptosis by stimulating the cell cycle-dependent kinase inhibitor p21 and by reducing cyclin D1 gene expression. The induction of p21 gene expression by PPARγ ligands may be mediated through increased Sp1- and NF-IL6 (C/EBP)-dependent transcriptional activation. These observations unveil a mechanism for p21 gene regulation in lung carcinoma that represents a potential target for therapy.

Introduction

Cell cycle progression is regulated by interactions between cyclins and cyclin-dependent kinases (1, 2). A family of negative cell cycle regulators, cyclin-dependent kinase inhibitors, modulates these events, especially those controlling the transition of G1 to S-phase (2). The latter includes two families, the CIP/KIP family and the INK4 family. p21 is a member of the CIP/KIP family (2, 3) and plays a crucial role in growth arrest induced in transformed and normal cells by a variety of mechanisms (4). p21 was identified and cloned in a number of cellular systems including p53-dependent growth arrest and DNA-damage induction (5–7), p53-independent melanoma (mda-6; 8, 9), leukemia cell differentiation (10, 11), and during cellular senescence (sdi-1) in human skin fibroblasts (12). p21 is poorly expressed in quiescent cells; it is rapidly induced when cells are stimulated with mitogens, and its expression then declines as cells reach mid-late G1 phase (2, 13). Although many studies have implicated p21 in cell cycle regulation, especially in G1 arrest, the clinical significance of p21 in human lung carcinoma is still controversial (14). However, in patients with non-small cell lung carcinoma, p21 expression was shown to be an independent prognostic factor (15, 16), and it may represent an important clinical marker of outcome in non-small cell lung carcinoma. We believe that p21 is a potent modulator of lung carcinoma cell growth as well as an important marker in the prognosis of this disease. It is for this reason that we have focused our attention on this cell cycle gene and how its ex-
pression is affected by peroxisome proliferator-activated receptor (PPAR) ligands.

The PPAR\(\gamma\) is a member of the nuclear receptor superfamily of ligand-dependent transcriptional factors (17). PPAR\(\gamma\) plays an important role in the differentiation of adipocytes and monocytes/macrophages, as well as in cell proliferation, apoptosis, and carcinoma cell arrest (17). In humans, PPAR\(\gamma\) expression is detectable in normal tissues including brain, breast, prostate, colon, lung, ovary, and placenta (18–21). Recent studies revealed that PPAR\(\gamma\) ligands can cause growth inhibition by inducing the differentiation or by promoting the apoptosis of several leukemia cells and solid tumors including breast, prostatic, colorectal, gastric, and lung carcinoma (20, 22–25). Of note, modulation of p21 expression by PPAR\(\gamma\) ligands has been shown in several cell types (26–28). However, the mechanisms by which PPAR\(\gamma\) activation regulates G1 phase arrest and p21 gene expression remain to be elucidated.

Herein, we show that PPAR\(\gamma\) ligands inhibit human lung carcinoma cell growth and induce apoptosis by stimulating the expression of the cyclin-dependent kinase inhibitor p21 and by reducing the expression of cyclin D1. These results suggest that the induction of p21 by PPAR\(\gamma\) ligands has been shown in several cell types (26–28). However, the mechanisms by which PPAR\(\gamma\) activation regulates G1 phase arrest and p21 gene expression remain to be elucidated.

Materials and Methods

Culture and Chemicals. The human small cell lung carcinoma cell lines (H345, H2081) and non-small cell lung carcinoma cell lines (H1838, H2106) were obtained from the American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, HEPES buffer, 50 IU/ml penicillin/streptomycin, and 1 \(\mu\)g of amphotericin (complete medium) as described previously (29). The PGJ2 and ciglitazone (Cig) were purchased from Alexis Biochemical (San Diego, CA). The PPAR\(\gamma\) antagonist GW9662 (30) was obtained from Cayman Chemical Co. (Ann Arbor, MI). The dual-luciferase report assay kit, the gel shift assay system and 5\(\text{'}\)-[\(\text{^32}\)P]ATP was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). TACS Apoptotic DNA Laddering Kit were performed in the Cepheid Smart-Cycler real-time PCR cycler (Sunnyvale, CA; Ref. 34). Experiments were performed in triplicate for each data point.

Real-Time RT-PCR. This procedure, which was based on the time point during cycling when amplification of the PCR product was first detected, rather than on the amount of PCR product accumulated after a fixed number of cycles, was described previously (34). Final results were expressed as \(n\)-fold differences in p21, or cyclin D1 gene expression relative to the glyceraldehyde-3-phosphate dehydrogenase gene. All PCR reactions using LightCycler-FastStart DNA Master SYBR Green I kit were performed in the Cepheid Smart-Cycler real-time PCR cycler (Sunnyvale, CA; Ref. 34). Experiments were performed in triplicate for each data point.

Detection of DNA Fragmentation. Genomic DNA was isolated from human lung carcinoma cells using TACS Apoptotic DNA Laddering Kits (R&D Systems) to evaluate apoptotic cell death. Procedures for DNA isolation and detection followed the protocol supplied by the product supplier as we described before (29). After running in a 1.5% TreviGel 500 gel [% (w/v)] in 1X Tris-acetate-EDTA, DNA was visualized with ethidium bromide staining under a UV transilluminator and photographed.

Oligodeoxynucleotide Transfections. Phosphorothioate p21 antisense and random sequence control oligodeoxynucleotides (ODN) designed around the translation start site were synthesized by Sigma Genosys according to published data (35). The p21 antisense sequence was 5\(\text{'}\)-GAC ATCACGGATC-GGACAT-3\(\text{'}\). The random sequence control was 5\(\text{'}\)-CTGATGACATCCCGAC-3\(\text{'}\). The antisense sequence was 5\(\text{'}\)-CCATGGAGAAGGCTGGG-3\(\text{'}\), antisense (5\(\text{'}\)-GGTTCGAGGCTGAG-3\(\text{'}\)). For the transfection procedure, cells were seeded in 6-well culture plates and incubated with various concentrations of PGJ2, Cig, or WY14643 for up to 4 days. The cells were subsequently harvested by trypsinization using trypsin/EDTA and counted under the microscope after trypan-blue staining.

Reverse Transcriptase (RT)-PCR. Total RNA was prepared from human lung carcinoma cells with TRIzol Reagent (Life Technologies, Inc., Rockville, MD) according to the manufacturer’s instructions. To amplify 474 bp PPAR\(\gamma\), 354 bp p21, 323 bp cyclin D1, and 200 bp glyceraldehyde-3-phosphate dehydrogenase cDNA fragments, the sequences of PCR primers (Sigma Genosys, Woodlands, TX) were as follows: for PPAR\(\gamma\) sense (5\(\text{'}\)-CTCTCCGAATGGGACACC-3\(\text{'}\)), antisense (5\(\text{'}\)-GC-ATTATAGACATCCCGAC-3\(\text{'}\)); for p21 sense (5\(\text{'}\)-GGCATG-GGAACCTTGACATTGT-3\(\text{'}\)), antisense (5\(\text{'}\)-GGGGCTTCTTTTGGAGAAAT-3\(\text{'}\)); for cyclin D1 sense (5\(\text{'}\)-GGCAACCGGAGGTCGCG-3\(\text{'}\)), antisense (5\(\text{'}\)-GTGCGTGGTAGATGTAGCCAA-3\(\text{'}\)), and for glyceraldehyde-3-phosphate dehydrogenase sense (5\(\text{'}\)-CCATGGAGAAGGCTGGG-3\(\text{'}\)), antisense (5\(\text{'}\)-CAAGTTGTCACTGGATGACC-3\(\text{'}\)) according to published data (31–33). RT-PCR was carried out as described previously (31). The samples were first denatured at 95°C for 30 s, followed by 32 PCR cycles, each with temperature variations as follows: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The last cycle was followed by an additional extension incubation of 7 min at 72°C. Analysis of amplicons was accomplished on 1% agarose gel containing 0.2 \(\mu\)g/\(\mu\)l ethidium bromide and visualized under UV transilluminator. The densitometric analysis of PCR products was performed by computer software (Bio-Rad Quantity One), GS-800 Imaging Densitometer (Bio-Rad, Hercules, CA), and standardized to the glyceraldehyde-3-phosphate dehydrogenase product.

Cell Growth Inhibition Assay. H2106 human lung carcinoma cells (10\(^6\) cells/well) were seeded in 6-well culture plates and incubated with various concentrations of PGJ2, Cig, or WY14643 for up to 4 days. The cells were subsequently harvested by trypsinization using trypsin/EDTA and counted under the microscope after trypan-blue staining.
PPARγ ligands for 24 and 48 h for Western Blot and cell growth assay, respectively.

**Western Blot Analysis.** The procedure was performed as described previously (36). Protein concentrations were determined by the Bio-Rad protein assay. Equal amounts of protein (50 μg) from whole cell lysates were solubilized in 2× SDS-sample buffer, separated on SDS polyacrylamide gels. The separated proteins were transferred onto nitrocellulose and blocked with BLOTTO (1× Tris-buffered saline [10 mM Tris HCl (pH 8.0), 150 mM NaCl]) with 5% nonfat dry milk and 0.1% Tween 20 for overnight at 4°C. Blots were incubated with antibodies raised against rabbit p21 (1:500), cyclin D1, or PPARγ (1:1000 dilution) for 2 h at room temperature, then incubated with a secondary antibody raised against rabbit IgG conjugated to horseradish peroxidase (1:10,000 dilution; Sigma) for 1 h at room temperature. The blots were washed, transferred to freshly made enhanced chemiluminescence solution (Amer sham, Arlington, IL) and exposed to X-ray film. Protein bands were quantified by densitometric scanning using a Bio-Rad GS-800-calibrated densitometer. In controls, the p21, cyclin D1, and PPARγ antibodies were omitted or replaced by serum IgG.

**Plasmids.** The 2.3-kb fragment of the p21 promoter construct ligated to firefly luciferase reporter gene has been reported previously (37). Synthetic Renilla luciferase report vector (phRL-SV40) was obtained from Promega.

**Transient Transfection Assays.** Human lung carcinoma cells were seeded at a density of 1 × 10^5 cells/well in 6-well dishes and grown to 60% confluence. For each well, the plasmid DNA containing wild-type p21 promoter construct, and 0.2 μg of the internal control plasmid phRL-SV40 (Renilla luciferase gene) were cotransfected into the cells using 6 μl of FuGENE 6 lipofection reagent as described in our earlier work (38). After 24 h of incubation, cells were treated with PPARγ ligands and PPARα ligand for an additional 24 h. The preparation of cell extracts and measurement of luciferase activities were carried out using the dual-luciferase reporter kit according to recommendations by the manufacturer. The assays for firefly luciferase activity and Renilla luciferase activity were performed sequentially using two reaction tubes in a luminometer with two injectors (Thermo Labsystems, Helsinki, Finland). Changes in firefly luciferase activity were calculated and plotted after normalization with changes in Renilla luciferase activity in the same sample.

**Electrophoretic Mobility Shift Assay.** Nuclear protein extracts were prepared for electrophoretic mobility shift assay as described earlier (39). The protein content of the nuclear extract was determined using the Bradford protein assay kit (Sigma). Electrophoretic mobility shift assay experiments were performed as described previously (29). The probes of double-stranded oligonucleotides for Sp1 and NF-IL6 (C/EBP) that were synthesized by Sigma-Genosys based on human p21 promoter sequence (37, 40) were, as follows: wild-type Sp1 (5'-GGGGGTCCCGCTCCCTTTGA-3'); mutant Sp1 (5'-GGGGGTCT- gacctcctcttga-3'). Wild-type NF-IL6 (C/EBP; 5'-GTACT-TAAGAATAATGGAAT-3'); C/EBP mutant (5'-GTACCAA- GAATAATGGAAT-3'). The underlined bases indicate mutations. The complimentary oligonucleotides were annealed and purified following the manufacturer’s instructions. The Sp1 and NF-IL6 (C/EBP) oligonucleotides were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase as recommended by the manufacturer. Ten μg nuclear proteins from control and treated cells were incubated with 32P-labeled oligonucleotide probe under binding conditions (Promega) for 20 min at room temperature in a final volume of 20 μl. For cold competition, a 100-fold excess of the respective unlabeled consensus oligonucleotides was added in reaction buffer contained nuclear protein for 10 min before adding probe. The same amount of mutated oligonucleotide probe or 100-fold excess of mutated oligonucleotides was used as another control. After binding, protein-DNA complexes were electrophoresed on a native 4.5% polyacrylamide gel using 1× Tris-glycine buffer. Each gel was then dried and subjected to autoradiography at −80°C.

**Statistical Analysis.** All experiments were repeated a minimum of three times. All data collected from electrophoresis gel mobility shift assays, luciferase activity assays, RT-PCR, or real-time RT-PCR and Western blot were expressed as means ± SD. The data presented in some figures are from a representative experiment, which was qualitatively similar in the replicate experiments. Statistical significance was determined with Student’s t test (two-tailed) comparison between two groups of data sets. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition (P < 0.05, see Fig. legends).

**Results.**

**PPARγ Gene Expression in Human Lung Carcinoma Cells.** To confirm that PPARγ is expressed in the lung carcinoma cell lines studied, we examined its expression by RT-PCR and Western blot analyses (Fig. 1). Two human small cell lung carcinoma cell lines (H345, H2081) and two non-small cell lung carcinoma cell lines (H2106, H1838) contained PPARγ mRNA...
Regulation of p21 through PPARγ Signaling

Con when compared with the untreated cells.

A
determined at the indicated time period. Data are expressed as mean ± SD of three independent experiments. *, P < 0.05 when compared with the untreated cells.

![Graph A](image)

**Fig. 2** Dose-dependent effect of PGJ2 and ciglitazone (Cig) on human lung carcinoma cell growth. H2106 cells were treated with indicated concentrations of either PGJ2 (A) or Cig (B), and cell numbers were determined on day 4. Data are expressed as mean ± SD of three independent experiments. *, P < 0.05 when compared with the untreated cells.

![Graph B](image)

B
determined on day 4. Data are expressed as mean ± SD of three independent experiments. *, P < 0.05 when compared with the untreated cells.

![Graph C](image)

**Fig. 3** Time-dependent inhibition of human lung carcinoma cell growth. H2106 cells were treated with solvent control, 30 μM ciglitazone (Cig), 20 μM PGJ2 or 5 μM WY14643, and cell numbers were determined at the indicated time period. Data are expressed as mean ± SD of at least three independent experiments. *, indicated P < 0.05 when compared with the untreated cells. Con, control.

with variable levels of expression (Fig. 1A). PPARγ protein was also detected in all cell lines studied (Fig. 1B). As expected, phorbol 12-myristate 13-acetate and PGJ2 enhanced the expression of PPARγ mRNA as shown in H1838 cells (Fig. 1C).

**Effect of PPARγ Ligands on Proliferation of Human Lung Carcinoma Cells.** Next, we examined the effect of PPARγ activation on cell growth in H2106 cells that expressed high levels of PPARγ. As shown in Fig. 2, the PPARγ ligands PGJ2 (A) and Cig (B) inhibited the growth of these cells in a dose-dependent manner. Statistical significance was obtained at 10–30 μM of PGJ2 and Cig. Fig. 3 shows the time-dependent effects of these agents on H2106 cell growth. PGJ2 and Cig significantly inhibited cell proliferation when compared with untreated cells and cells treated with the PPARα agonist WY14643 starting at 48 h of treatment. The PPARγ ligands showed similar dose- and time-dependent activity on the growth of the other lung carcinoma cell lines (H1838, H2081, and H345; data not shown). The suppressive cell growth effects of relatively low doses of either PGJ2 (10 μM) or Cig (30 μM) for 72 h were reversible when the media was changed to fresh media containing no PPARγ ligands. However, the cell growth inhibition became irreversible after 72 h treatment or when cells were exposed to higher doses of either PGJ2 (20 μM) or Cig (50 μM; not shown).

**Effect of PPARγ Ligands on Induction of Human Lung Carcinoma Cell Apoptosis.** In Fig. 3, we demonstrated that PPARγ ligands not only inhibited lung carcinoma cell proliferation, but they also reduced the number of cells below the baseline. This suggested the possibility of induction of apoptosis. To test this possibility, we evaluated PPARγ-treated cells for DNA fragmentation as a marker for cells undergoing apoptosis. H1838 cells showed significant DNA fragmentation after 72 h treatment with Cig when compared with the control (Fig. 4A, Lane 2 versus 1). In H2061 cells, the PPARα ligand WY14643 (25 μM), unlike PGJ2, did not induce apoptosis (Fig. 4B, Lane 2 versus 3). H345 and H2081 cells showed a similar apoptotic response when treated with Cig and PGJ2 (data not shown).

**Induction of p21 Gene Expression by PPARγ Ligands.** Having demonstrated that PPARγ ligands inhibit lung carcinoma cell growth and promote apoptosis, attention focused on the potential mechanisms mediating this effect. Others have demonstrated that PPARγ ligands induce cell cycle arrest and apoptosis by up-regulation of p21. To determine the relevance of this pathway in our system, we examined the effects of PPARγ ligands on p21 expression. Both PGJ2 and Cig significantly induced p21 mRNA levels in H1838 cells at 4 h as determined by RT-PCR (Fig. 5A, top) and confirmed by real-time RT-PCR (Fig. 5A, bottom). Cig and PGJ2 increased p21 expression 2.5- and 4-fold, respectively. In accordance with the changes observed in p21 mRNA, Western blot analysis revealed that PGJ2, Cig, troglitazone, and GW1929 (another PPARγ ligand; Ref. 41), induced the expression of p21 protein levels in H2106 cells after an 8-h culture (Fig. 5B).

We also examined the effect of PPARγ ligands on one of the cell cycle control genes, cyclin D1, and found that the
PPAR\(\gamma\)/H9253 ligands PGJ2 and Cig were found to significantly activate the 2.3-kb fragment of the p21 promoter, whereas WY14643 had little effect.

**PPAR\(\gamma\) Ligands Increase the Binding Activities of Sp1 and NF-IL6 (C/EBP).** Next, electrophoretic mobility shift assay was performed to identify potential nuclear transcription factors that could mediate regulation of the p21 gene by the PPAR\(\gamma\) ligands. The human p21 promoter region contains multiple transcription factor binding sites, including Sp1 and binding motifs for NF-IL6 (C/EBP), and proteins that bind to these sites play important roles in cell cycle arrest and apoptosis in carcinoma cells (37, 40, 42, 43). In agreement with those studies, we found that H2106 cells treated with PPAR\(\gamma\) ligands for

**PPAR\(\gamma\) Ligands Stimulate p21 Promoter Activity.** We demonstrated that PPAR\(\gamma\) ligands increased p21 gene expression in human lung carcinoma cells. To explore whether this effect reflects increased transactivation of the p21 promoter, transient transfections were performed with human p21 promoter constructs. As shown in Fig. 7, the PPAR\(\gamma\) ligands

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Fig. 5 Effect of peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) ligands on p21 expression. Total RNA and cellular protein was isolated from H1838 cells that were cultured for 4 and 8 h, respectively, in the presence or absence of indicated compounds [30 \(\mu\)M ciglitazone (Cig), 20 \(\mu\)M troglitazone (Trog), PGJ2 and GW1929]. Reverse transcription (RT)-PCR (Fig. 6A, top) and real-time RT-PCR (Fig. 6A, bottom) were performed. B, bottom, was obtained by densitometry. The bar graphs represent the mean ± SD of cyclin D1/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or p21/actin of at least three independent experiments. * significant differences as compared with the vehicle control. Con, control.

Fig. 6 Effect of peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) ligands on cyclin D1 gene expression in human lung carcinoma cells. Total RNA and cellular protein were isolated from H2106 cells that were treated with PGJ2, troglitazone (Trog; 20 \(\mu\)M), or ciglitazone (Cig; 30 \(\mu\)M) for 12 and 24 h, respectively. Reverse transcription (RT)-PCR (A, top), real-time RT-PCR (A, bottom) and Western blot analysis (B) were performed using cyclin D1 primers and polyclonal antibodies against cyclin D1, respectively. A, bottom, indicates fold changes of cyclin D1 mRNA levels by PPAR\(\gamma\) treatment as compared with control. The bar graphs represent the mean ± SD cyclin D1/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or cyclin D1/actin band densities of at least three independent experiments. * significant differences as compared with the vehicle control. Con, control.
24 h showed an induction in Sp1 (Fig. 8A) and NF-IL6 (C/EBP; Fig. 8B) binding activities. There was no binding activity when the Sp1 or NF-IL6 (C/EBP) sites were mutagenized. The PPARγ agonist WY14643 had little effect, suggesting the specificity of the activity of the PPARγ ligands. In competition assays, we found significant attenuation of Sp1 and NF-IL6 (C/EBP) bands by adding a 100-fold molar excess of unlabeled oligonucleotides [Sp1 and NF-IL6 (C/EBP)]. These results confirm that the PPARγ ligands (PGJ2 and Cig) can increase the binding levels of Sp1 and NF-IL6 (C/EBP) transcription factor complexes in human lung carcinoma cells.

**Fig. 7** Effect of peroxisome proliferator-activated receptor γ (PPARγ) ligands on p21 promoter activity. H1838 human lung carcinoma cells were cotransfected with p21 promoter construct ligated to luciferase and 0.2 μg of control Renilla hpRL-SV40 plasmid. After transfection for 24 h, cells were cultured in the presence or absence of indicated compounds [20 μM PGJ2, 30 μM ciglitazone (Cig), or 25 μM WY14643] for another 24 h, after which luciferase activity was determined using dual-report luciferase kit. A representative experiment of at least three performed is shown. *, significant difference from control. Con, control.

**Discussion**

PPARγ ligands have been implicated in the regulation of cell differentiation, apoptosis, and cell cycle control (18). The expression of PPARγ and the effect of PPARγ ligands on cell growth inhibition and apoptosis have been investigated in many cells including lung carcinoma (21, 23–26). Although these studies showed that several PPARγ ligands can induce cell growth inhibition and apoptosis, little is known about the mechanism(s) mediating these effects on human lung carcinoma cells. Therefore, we investigated whether the cell cycle control genes cyclin D1 and the cyclin-dependent kinase inhibitor p21 are involved in PPARγ-mediated growth regulation in these cells. We found variable levels of PPARγ mRNA and protein in four human lung carcinoma cell lines. Treatment with the PPARγ

**Fig. 8** Electrophoretic mobility shift assay (EMSA) to determine the Sp1 and CAAT/enhancer binding protein (C/EBP) protein binding activities in p21 promoter affected by peroxisome proliferator-activated receptor γ (PPARγ) ligands. Oligonucleotides containing the Sp1 (A) or C/EBP (B) sites were end labeled with [γ-32P]ATP and incubated with nuclear extracts (10 μg) from H2106 cells treated with PPARγ ligands PGJ2 (20 μM), ciglitazone (Cig; 30 μM), or PPARα ligand WY14643 for 24 h. For competition assays, a molar excess (×100) of consensus Sp1 or C/EBP oligonucleotide was added to the binding reaction. Oligonucleotides containing a mutated Sp1 (Cold Sp1) or C/EBP site (Cold C/EBP) that were end labeled with [γ-32P]ATP or 100 times molar excess of unlabeled mutated Sp1 oligonucleotide (mut Sp1) were used to confirm the binding specificity. Nuclear extract from HeLa cells was used as positive control for Sp1. Con, control; FP, free probe.
ligand PGJ2 induced PPARγ mRNA levels as compared with the control, suggesting that PPARγ signaling was functional. We next examined the effect of PPARγ activation on cell growth in these cells. Treatment with PGJ2 and Cig suppressed cell growth in a dose- and time-dependent manner. Of note, we found that the PPARγ ligands PGJ2, troglitazone, and Cig, but not the PPARα agonist WY14643, induced apoptosis of H2106 or H1838 cells. These results expand current knowledge about the proapoptotic effects of PPARγ ligands on human lung carcinoma cells (13, 17).

Expression of cyclin-dependent kinase inhibitors is purported to play important roles in the control of cell cycle in many cell types, including lung carcinoma in vitro and in vivo (45–47). Although the exact mechanism of growth inhibition of carcinoma cells by PPARγ ligands is not known, it has been shown to be associated with alterations in the expression of the cell cyclin-dependent kinase inhibitor p21 (26, 27). Consistent with this idea, we showed that PGJ2 and Cig up-regulated the expression of p21 protein and mRNA concomitant with inhibition of cyclin D1 protein and mRNA levels. Inhibition of cyclin D1 gene expression through PPARγ signaling had also been reported in other cell types as well as lung carcinoma cells (48, 49). As such, our results support the hypothesis that suppression of lung carcinoma cell growth through PPARγ activation may be mediated via reduction of cyclin D1 levels.

p21 has been shown to be regulated at the level of transcription in different cell types. Park et al. (37) showed that mitogen-activated protein kinase signaling increased p21 promoter activity via multiple transcription factors. Li et al. (50) demonstrated that growth inhibition associated with an increase in JunD/AP-1 activity in normal intestinal epithelial cells was at least partially mediated through the activation of the p21 promoter. To investigate whether PPARγ ligand-mediated up-regulation of p21 reflects transactivation of the promoter, we performed transient transcription experiments using a p21 promoter reporter construct. We found that the PPARγ ligands PGJ2 and Cig increased p21 promoter activity, whereas the PGJ2/Cig antagonsist (55) who showed that antisense ODN for p21 antisense ODN for 24 h before exposing them to 30 μM ciglitazone (Cig) or 20 μM PGJ2 for an additional 48 h as indicated, and cell numbers were determined. Data are expressed as mean ± SD of at least three independent experiments. *, significant difference from control. **, significance of combination treatment as compared with PGJ2 or Cig alone. Con, control.  

**Fig. 10** Antisense p21 oligodeoxynucleotides (ODNs) antagonized peroxisome proliferator-activated receptor γ (PPARγ) ligand-mediated suppression of carcinoma cell proliferation. H2106 cells were treated with either solvent control or transfected with control ODN or p21 antisense ODN for 24 h before exposing them to 30 μM ciglitazone (Cig) or 20 μM PGJ2 for an additional 48 h as indicated, and cell numbers were determined. Data are expressed as mean ± SD of at least three independent experiments. *, significant difference from control. **, significance of combination treatment as compared with PGJ2 or Cig alone. Con, control.

**Fig. 9** Antisense p21 oligodeoxynucleotide (ODN) prevents peroxisome proliferator-activated receptor γ (PPARγ) ligand-induced p21 protein levels in human lung carcinoma cells. H1838 cells were treated with solvent control or control and p21 antisense ODNs for 24 h before adding PGJ2 (20 μM) or ciglitazone (Cig; 30 μM) for additional 24 h, then subjected to Western blot analysis. Actin was used as internal control for normalization purposes (A). The bar graphs (B) obtained by densitometry represent the mean ± SD p21/actin of at least three independent experiments. *, significant difference from control. **, significance of combination treatment as compared with PGJ2 or Cig alone. Con, control.
carcinoma cells was abolished by stable transfection with a p21 antisense construct.

Taken together, our study indicates that PPARγ ligand inhibition of human lung carcinoma cell growth and induction of apoptosis are mediated via up-regulation of the cyclin-dependent kinase inhibitor p21 and reduction of cyclin D1 gene expression. Induction of p21 gene expression by PPARγ ligand appears to be, at least in part, regulated through C/EBP and Sp1 sites in the p21 gene promoter. Although additional studies will be required to determine the clinical implications of our findings, our observations suggest that ligand activation of PPARγ represents a potential new molecular target for effective therapy against human lung carcinoma. Studies in humans, animals, and in cultured cells support the idea that modulation of PPAR-γ activation may have therapeutic benefits in other tumors including colon and pituitary carcinoma (57, 58). There was no difference in survival between patients who received adjuvant chemotherapy and those who did not (59). We speculate that specific PPARγ ligands used as single agents or as part of combination regimens could serve to further improve outcomes in these patients by inhibiting carcinoma cell growth.

References
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