Journal of Cellular Biochemistry 96:760–774 (2005)

Activation of Peroxisome Proliferator-Activated Receptor-γ by Troglitazone (TGZ) Inhibits Human Lung Cell Growth

Mingyue Li,[1] Tak W. Lee,[1] Tony S.K. Mok,[2] Timothy D. Warner,[3] Anthony P.C. Yim,[1] and George G. Chen[1]*

[1]Department of Surgery, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China
[2]Department of Clinical Oncology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China
[3]The William Harvey Research Institute, Barts and the London, Queen Mary University of London, London, United Kingdom

Abstract Peroxisome proliferator-activated receptor-gamma (PPAR-γ) is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors and a crucial regulator of cellular differentiation. PPAR-γ ligands have been demonstrated to inhibit growth of several cancer cells. In this study, two human lung cancer cells (NCI-H23 and CRL-2066) and one human lung normal cell (CRL-202) were used for the experiments. The results showed that in consistence with the loss of viability, troglitazone (TGZ) induced apoptosis of CRL-2066 and NCI-H23 cells but not CCL-202 cells. TGZ upregulated PPAR-γ expression in all the three lung cell lines, especially in the cancer cells. In association of the time-dependent inhibition of the cell proliferation, TGZ downregulated the expression of Bcl-w and Bcl-2 but activated extracellular signal-regulated kinase (ERK)1/2 and p38, suggesting that the growth-inhibitory effect of TGZ is associated with the reduction of Bcl-w and Bcl-2 and the increase of ERK1/2 and p38 activation. SAPK/JNK activation assay showed a decreased activity in all the three cell lines tested after TGZ treatment. It was also demonstrated that TGZ could activate PPAR-γ transcriptionally. We conclude that TGZ inhibits growth of human lung cancer cells via the induction of apoptosis and the inhibition of cell growth, at least in part, in a PPAR-γ-relevant manner. The mechanism of TGZ is associated with the activation of ERK and p38, the reduction of SAPK/JNK activity, and the alteration of Bcl-w and Bcl-2. J. Cell. Biochem. 96: 760–774, 2005.

Key words: apoptosis; lung cancer; peroxisome proliferator-activated receptor-gamma; troglitazone

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptors superfamily of ligand-activated transcriptional factors that include receptors for steroids, thyroid hormone, vitamin D, and retinoid acid [Mangelsdorf et al., 1995]. PPAR binds to peroxisome proliferator responsive element (PPRE) as a heterodimer with the retinoid X receptor (RXR) [Gearing et al., 1993]. To date, three PPAR subtypes have been isolated and are termed as PPAR-α, PPAR-β (also called δ, NUC-1, or FAAR), and PPAR-γ [Mangelsdorf et al., 1995; Vamecq and Latruffe, 1999]. PPAR-α is a key regulator of lipid metabolism. Specifically, activation of PPAR-α results in proliferation of peroxisomes and the induction of gene involved in β-oxidation of fatty acids [Desvergne and Wahli, 1999]. PPAR-β is ubiquitously expressed and has been implicated in the repression of PPAR-α and -γ transcriptional activity [Shi et al., 2002] as well as in wound healing [Di-Poi et al., 2002]. PPAR-γ is highly expressed in adipose tissue and has a dominant regulatory role in adipocyte differentiation [Tontonoz et al., 1994; Spiegelman and Flier, 1996]. In addition to adipogenic and

Grant sponsor: Earmarked Research Grant from the Hong Kong Research Grants Council; Grant number: CUHK 4390/03M.

*Correspondence to: George G. Chen, Department of Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong, China. E-mail: gchen@cuhk.edu.hk

Received 12 January 2005; Accepted 22 February 2005
DOI 10.1002/jcb.20474

anti-inflammatory effects, PPAR-γ activation has been shown to be anti-proliferative by virtue of its differentiation-promoting effect, suggesting that activation of PPAR-γ might be useful in slowing or arresting the proliferation of dedifferentiated tumor cells. In humans, PPAR-γ is expressed in multiple types of tissues, including the breast, colon, lung, ovary, and placenta [Lambe and Tugwood, 1996; Elstner et al., 1998; Mueller et al., 1998; Sarraf et al., 1998]. A number of PPAR-γ ligands have been identified, including natural prostaglandins, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2), and the synthetic anti-diabetic thiazolidinediones (TZD), such as troglitazone (TGZ), and certain polyunsaturated fatty acids. PPAR-γ ligands are able to bind to the PPAR-γ transcription factor, which then forms a heterodimeric complex with RXR that functions as a central regulator of differentiation, and a modulator of cell growth. There is increasing evidence showing anti-tumorigenic activity of PPAR-γ ligands [Chang and Szabo, 2000; Tsubouchi et al., 2000; Wakino et al., 2001; Yang and Frucht, 2001; Yin et al., 2001; Masamune et al., 2002; Keshamouni et al., 2004]. In addition, naturally occurring somatic mutation in the gene encoding PPAR-γ has been found in a proportion of sporadic colorectal carcinomas [Sarraf et al., 1999]. A dominant-negative PPAR-γ mutant with substitution at L468A/E471A inhibits PPAR-γ-mediated adipogenesis [Gurnell et al., 2000] and colorectal cancer cells growth arrest [Chen et al., 2003]. These findings further support that a functional and non-mutated PPAR-γ are necessary for its anti-tumor activity.

Lung cancer, the major cause of cancer deaths in the world, can be divided into two broad groups: small cell lung cancer (SCLC) accounts for 20–25% of bronchogenic carcinomas and non-small cell lung cancer (NSCLC) constitutes almost all of the remainder [Parkin et al., 1993; Minna, 1998]. Although it is still unclear whether SCLC and NSCLC are derived from the same or different stem cells, there has been much progress made towards understanding the molecular etiology of lung cancer, particularly involving mutations in oncogenes and tumor suppressor genes (recessive oncogenes). The epidemic of lung cancer is directly attributable to cigarette and therefore major efforts are needed to prevent initiation and aid with cessation of smoking. The average 5-year survival rate for localized and metastatic lung cancer is 48% and 2.5%, respectively [Feng et al., 2001; Gargiullo et al., 2002]. With only 15% of all patients are diagnosed at an early, localized stage because lung cancer begins to grow silently without any symptoms until the cancer has developed into in an advanced stage [Gargiullo et al., 2002]. There is also currently no accepted adjuvant or palliative treatment modalities that have been conclusively shown to prolong survival in lung cancer [Feng et al., 2001]. New methods are needed for early detection, treatment, and prevention of this disease. Cancer cells have been shown to dedifferentiate prior to acquiring proliferative and anti-apoptotic phenotype [Bloch, 1984; Pierce and Speers, 1988]. Therefore, the induction and maintenance of a differentiated state has been an important strategy in the search for cancer therapeutics. The expression of PPAR-γ is often increased in human primary tumors, compared to corresponding normal tissue by both immunochemical staining and Western blotting [Keshamouni et al., 2004]. PPAR-γ is known to express in both human SCLC cells and NSCLC cells [Tsubouchi et al., 2000; Keshamouni et al., 2004]. And PPAR-γ ligands, such as TGZ and 15-deoxy-$\Delta^{12,14}$-PGJ_2, inhibit the growth of human lung cancer cells through ligand-induced growth arrest and induction of apoptosis [Tsubouchi et al., 2000; Satoh et al., 2002; Wick et al., 2002; Han and Roman, 2004; Keshamouni et al., 2004]. These results raise the possibility that PPAR-γ may play role in the pathogenesis and progression of human lung cancer and that PPAR-γ agonists may serve as anti-tumor agents against lung cancer. Since induction of differentiation is a relatively nontoxic and therapeutic approach, PPAR-γ ligands, such as TGZ and 15-deoxy-$\Delta^{12,14}$-PGJ_2, may be candidates for a novel approach to the treatment of human lung cancer.

Recent study has demonstrated that apoptosis induced by activation of PPAR-γ was associated with decreased Bcl-2 and NF-κB in human colon cancer [Chen et al., 2002, 2003] and in human neuroblastoma cell [Kim et al., 2003]. Members of the Bcl-2 protein family are key regulators of cell death [Adams and Cory, 1998]. Mammalian Bcl-2 and its closest relatives, such as Bcl-X_L and Bcl-w, Mcl-l, A1, promote cell survival and their absence, such as that observed in mice lacking Bcl-w, results in tissue degeneration [Print et al., 1998],

whereas other members, such as Bax, Bid, and Bak and Bad, promote cell death [Print et al., 1998]. It is reported that activation of PPAR-γ triggers sustained extracellular signal-regulated kinase (ERK)1/2 activation and induces cell growth arrest/differentiation via a hitherto unknown mechanism [Kim et al., 2003; Keshamouni et al., 2004]. It has been known that the MAP kinase family of proteins, known as c-Jun NH_2-terminal kinase (JNK), ERK, and p38 MAP kinase, play an important role in cell survival and apoptosis. The activation of mitogen-activated protein kinase (MAPK) kinase pathway may exert either anti-apoptotic or pro-apoptotic influence depending on the cellular context [Ravi et al., 1998; Mackeigan et al., 2000; Krueger et al., 2001; Yu et al., 2001; Keshamouni et al., 2002, 2004; Kim et al., 2003]. Therefore, studies on the inhibitory effect of PPAR-γ ligands on cell proliferation suggest that it is reasonable to explore PPAR-γ ligands for the treatment of cancer. However, the precise mechanism responsible still needs to be further investigated.

Among PPAR-γ ligands, the anti-tumorigenic activity of TGZ has been well established. For example, TGZ significantly inhibits the growth of human lung cancer cells, human colorectal cells (HCT-116), human breast cancer (MCF-7), and human prostate cancer cells (PC-3) in immunodeficient mice [Elstner et al., 1998; Kubota et al., 1998; Sarraf et al., 1998; Tsubouchi et al., 2000; Keshamouni et al., 2004]. However, the molecular mechanism responsible for the anti-tumor effect of TGZ is not clearly known.

In the present study, we have demonstrated that the activation of PPAR-γ by TGZ in lung cells resulted in growth inhibition due to cell cycle arrest and apoptosis. The apoptotic pathway was involved in the alteration of Bcl-w, Bcl-2, ERK1/2, p38, and SAPK/JNK.

MATERIALS AND METHODS

Cell Culture

The human lung cell lines CCL-202, NCI-H23, CRL-2066 were cultured as the following description. Histopathologically, CCL-202 is the normal lung fibroblast cell, CRL-2066 is the small cell lung carcinoma, and NCI-H23 is the NSCLC cell. CCL-202 and NCI-H23 cells were grown in 100-mm tissue culture disk (Iwaki, Scitech Div. Asahi Techno. Glass, Chiba, Japan) in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (Gibco), 100 U/ml of penicillin, and 100 mg/ml of streptomycin (Gibco), in a humidified 5% CO2 atmosphere at 37°C. CRL-2066 was grown in Waymouth's MB 752/1 medium (Gibco) supplemented with 10% FBS (Gibco), 100 U/ml of penicillin, and 100 mg/ml of streptomycin (Gibco) in a humidified 5% CO_2 atmosphere at 37°C. The media were changed every 3 days, and the cells were separated via trypsinization using 0.05% trypsin/0.53 mM EDTA (Gibco) when they reached subconfluence.

RT-PCR

Total RNA was isolated from human lung cell lines by RNeasy Mini Kit (QIAGEN, GmbH, Hilden, Germany). We performed RT-PCR procedure to determine PPAR-γ mRNA expression using Reverse Transcription System (Promega, Madison, WI). In short, total RNA was used as a template for DNA synthesis according to the manufacturer's instructions. PCR was performed with each cDNA, PPAR-γ and G3PDH primers, and *Taq* DNA polymerase (Roche Diagnosstics GmbH, Mannheim, Germany). We used G3PDH mRNA as an internal control. The primers used were as follows: PPAR-γ sense primer, 5′-GCTGTCATTATTCTCAGTGGAGACC, PPAR-γ anti-sense primer, 5′-CAACTGGAAGAAGGGAAATGTTGG; G3PDH sense primer, 5′-CACTGCCACCCAGAAGACTG, G3PDH anti-sense primer, 5′-TCCACCACCCTGTTGCTGTA. The primer sets yielded PCR products of 314 and 435 bp for PPAR-γ and G3PDH, respectively. Reactions were incubated in an DNA Thermal cycler (Model GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA) at 94°C, 3 min followed by 32 cycles of denaturation 30 s, 94°C; annealing 30 s, 53°C; extension 30 s, 72°C, and the final extension at 72°C, 7 min. PCR products were run on a 2% agarose gel in TBE buffer, and visualized by ethidium bromide staining.

Western Blotting

In brief, Cells were homogenized with RIPA lysis buffer (1× PBS, 1%Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, add proteinase inhibitors before use). An equal amount of proteins (50 μg) was separated on a SDS/polyacrylamide gel and then transferred

onto a nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Inc., Little Chalfon, Germany). The goat polyclonal antibody against PPAR-γ (1:500), rat monoclonal antibody against Bcl-w (1:100) (Oncogene, San Diego, CA), mouse monoclonal antibody against Bcl-2 (1:400) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phospho-ERK1/2(1:400) (Santa Cruz Biotechnology, Inc.), mouse monoclonal antibody against phospho-p38 (1:2,000) (Cell Signaling Technology, Beverly, MA.), mouse monoclonal antibody against Phospho-SAPK/JNK (1:2,000) (Cell Signaling Technology, Beverly, MA), and goat polyclonal antibody against actin (1:500) (Santa Cruz Biotechnology, Inc.) were used. The blot was then incubated with the corresponding horseradish peroxidase (HRP) conjugated mouse anti-goat immunoglobulin, goat anti-Rat immunoglobulin (Santa Cruz Biotechnology, Inc.), or Goat anti-Mouse immunoglobulin (Santa Cruz Biotechnology, Inc.). Immunoreactive proteins were detected with the enhanced chemiluminescence Western blotting detection system (ECL Western Blotting detection, Amersham Biosciences, Little Chalfont Buckinghamshire, England).

Cell Viability Assay

Human lung cells (10^4 cells/100 μl/well) were seeded in 96-well plates (Becton Dickson Labware, Franklin Lakes, NJ) and incubated overnight to allow cells to attach to the plate. Cells were incubated with TGZ (0–100 μM) for 24, 48, 72, and 120 h. Having added 10 μl MTT (5 mg/ml in PBS), cells were incubated for 4 h at 37°C in a humidified atmosphere, 5% CO_2. The medium was aspirated and cells solubilized in 200 μl dimethylsulphoxide (DMSO). The absorbance at 570 nm was measured using a reference wavelength of 630 nm with a microplate reader. Cell viability was presented as % of control culture conditions.

Cell Cycle Analysis

Human lung cells (6×10^4 cells/2 ml/well) were seeded in 6-well plates (Becton Dickson Labware, Franklin Lakes, NJ) and incubated overnight to allow cells to attach to the plate. Cells were incubated with TGZ (20 μM) for 0, 6, 24, 48, and 72 h. Cell cycle analysis by flow cytometry was performed by detaching cells from culture plates with trypsin, washing twice with PBS and fixing in 75% ethanol-PBS. After keeping cells in fixative overnight at 4°C, cells were centrifuged for 5 min at 3,000*g*. The cell pellet was resuspended and incubated for 30 min in 0.05 mg/ml propidium iodide (PI), 1 mM EDTA, and 0.1% Triton X-100, and 1 mg/ml RNAse A in PBS for 30 min at room temperature. The suspension was then analyzed by Becton Dickinson FACScan. The ratio of cells in the G0/G1, S, and G2/M phases of cell cycle was determined by their DNA content and was presented as % of 0 h control conditions.

Detection of Apoptosis

Human lung cells (6×10^4 cells/2 ml/well) were seeded in 6-well plates (Becton Dickson Labware, Franklin Lakes, NJ) and incubated overnight to allow cells to attach to the plate. Flow Diagram of APO-DIRECT™ APOPTOSIS ASSAY kit (Chemicon international, San Diego, CA) was used for this assay. In brief, cells were treated by 20 μM TGZ for 0, 12, and 24 h, respectively. The cell was suspended in 1% (w/v) paraformaldehyde in PBS, pH 7.4 at a concentration of $1-2 \times 10^6$ cells/ml. The cell suspension was then placed on ice for 30–60 min. After centrifuging cells for 5 min at 300*g*, the supernatant was discarded. The cell was washed in 5 ml of PBS and the cell pellet was resuspended in PBS in a tube by gently vortexing. The cell concentration was adjusted to $1-2 \times 10^6$ cells/ml in ice-cold 70% (v/v) ethanol and let them stand for a minimum of 30 min on ice prior to staining for apoptosis detection. Apoptosis was measured according to the protocol provided by the kit and the result was presented as % fold of 0 h control conditions.

Real-Time PCR

Cells were treated with TGZ (20 μM) for 0, 2, 8, and 24 h. Total RNA was isolated from human lung cell lines by RNeasy Mini Kit (QIAGEN, GmbH, Hilden, Germany). We performed reverse transcription procedure for PPAR-γ mRNA by Reverse Transcription System (Promega, Madison, WI). Primers for PPAR-γ and G3PDH were designed using BIOEDIT software and tested to ensure amplification of single discrete band with no primer-dimmers. Primer sequences were as follows: PPAR-γ forward, 5′-TCAGGGCTGCCAGTTTCG-3′ PPAR-γ reverse, 5′-GCTTTTGGCATACTCTGTGATCTC-3′ (61 bp product); PPAR-γ TaqMan® MGB Probe, 5′-FAM™ CGTGGAGGCTGTGCA-3′. G3PDH forward, 5′-TCATGGGTGTGAACCATGAGA-3′; G3PDH reverse, 5′-GCAGGAGGCATTGCT-

GATG-3′ (62 bp product); G3PDH TaqMan® MGB Probe, 5′-VIC™ ATGACAACAGCCT-CAAGA-3′. Real-time PCR was conducted using TaqMan® Universal PCR Mastermix (Applied Biosystems, Branchburg, NJ) by an ABI PRISM′ 7700 Sequence Detection System. Each reaction was run in triplicate and contained 2 μl of cDNA template along with 300 nM PPAR-γ or G3PDH primers and 200 nM PPAR-γ or G3PDH probe in a final reaction volume of 25 μl. Cycling parameters were 50°C for 2 min and 95°C for 10 min to activate DNA polymerase, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed using software provided by Applied Biosystems, and PPAR-γ mRNA was normalized to G3PDH mRNA and presented as fold of 0 h control conditions.

Transfection and Luciferase Activity Assay

Cells were seeded in 12-well plates at a density of 1×10^5cells/well and allowed to reach 60–70% confluence. The cells were transiently transfected with Lipofectamine (Invitrogene, Carlshad, CA) at a Lipofectamine/DNA ratio of 3:1 in the absence of serum with 1 μg/well of either the PPAR-γ-responsive reporter plasmid pFATP- (PPRE)3-tkLuc (FATP-derived PPARγ response element with firefly luciferase gene under the control of a thymidine kinase promoter) or pGL3 basic vector or pGL3-control vector. After 6 h of transfection, cells transfected with pFATP- (PPRE)3-tkLuc were washed and treated with 0, 0.1, 1, 5, 15, 20, and 40 μM TGZ in the presence of serum. Cells transfected with pGL3 basic vector were washed and treated with 20 μM TGZ. After 24 h, cells were lysed and the luciferase activity was assayed in the lysates using Promega's luciferase assay reagents according to the manufacturer's protocol.

Analysis of Bcl-2 Family mRNA by Ribonuclease Protection Assay (RPA)

Cells were treated with TGZ (20 μM) for 0, 2, 8, and 24 h. Total RNA was isolated from human lung cell lines by RNeasy Mini Kit (QIAGEN, GmbH, Hilden, Germany). The RPA was done using Multi-Probe RNase Protection Assay System (BD Biosciences Pharmingen, San Diego, CA) and performed according to the protocol provided by the manufacturer. Briefly, the multi-Probe Template Sets probe, hAPO-2b, was labeled using Non-Rad In Vitro Transcription Kit (BD Biosciences Pharmingen) according to the protocol supplied by the manufacturer. Ten micrograms of total RNA was resuspended in 8 μl hybridization buffer followed by the addition of 2 μl Biotin-16-UTP-labeled probe according to the protocol supplied by the manufacturer (RPA Kit, BD Biosciences Pharmingen). Following labeling, the samples was separated and analyzed by a 4.75% acrylamide sequencing gel prepared in 0.5 × TBE, and the positive signal was analyzed according to the ECL protocol supplied by the manufacturer (RiboQuant. Non-Rad Detection Kit, BD Biosciences Pharmingen).

Statistial Analysis

The data are presented as means ± SD for at least three separate determinations for each treatment group.

RESULTS

Expression of PPAR-γ in Human Lung Cell Lines

To determine whether PPAR-γ is present in human lung cell lines, we first assessed the expression of PPAR-γ in human lung cell lines by RT-PCR and Western blotting. We found that PPAR-γ was expressed in all the three cell lines tested (Fig. 1).

Effects of PPAR-γ Ligand-TGZ on Proliferation of Human Lung Cancer Cells

To investigate the effects of TGZ on the proliferation of human lung cancer cells, we analyzed cell proliferation using cell viability assay. Cells were treated with TGZ (0–100 μM). TGZ significantly reduced cell viability of CCL-202, CRL-2066, and NCI-H23 cells in a dose-dependent manner as determined by MTT assay (Fig. 2).

To examine whether the inhibitory effect observed in the cell growth assay reflected a delay or arrest of cells, cells were treated with TGZ (20 μM) for 0, 6, 24, 48, and 72 h. And the cell cycle progression was evaluated after PI staining and fluorescence-activated cell sorting analysis. In CCL-202 and NCI-H23, an increase in the portion of cells in the G1-G0 phase of the cell cycle with a corresponding decrease in cells in S and G2-M phases was observed upon treatment with TGZ. TGZ also arrested the CRL-2066 cell in the G0/G1 phase and G2/M phase of cell cycle accompanied by the reduction of cells in the S phase (Fig. 3).

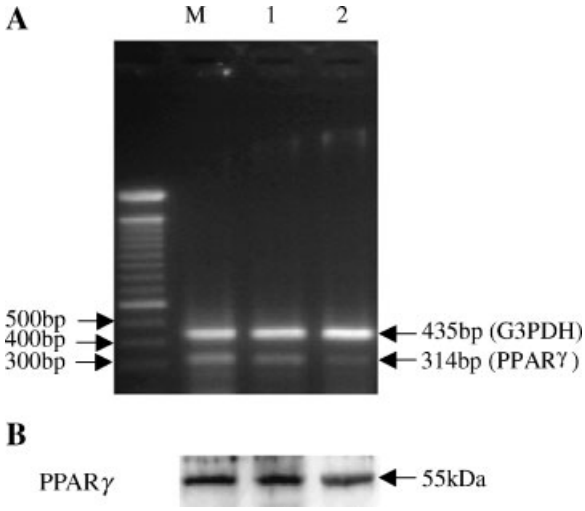


Fig. 1. Expression of PPARγ mRNA and protein in human lung cancer cell lines. **A**: RT-PCR. Total RNA were isolated from human lung cell lines (CCL-202, CRL-2066, NCI-H23), reverse transcribed using random primers, and amplified by PCR using specific primers for PPARγ or G3PDH. The RT-PCR products were electrophoresized on ethidium bromide-containing agarose gels. M: 100 bp ladder marker. **Lane 1**: CCL-202; (**lane 2**) NCI-H23; (**lane 3**) CRL-2066. G3PDH expected size: 435 bp, PPARγ expected size: 314 bp. **B**: Western blot analysis. Proteins were obtained from human lung cell lines CCL-202, NCI-H23, CRL-2066, separated on SDS–PAGE, probed with an anti-PPARγ antibody, and visualized with ECL detection kit as described in Materials and Methods.

The large number of DNA fragments appearing in apoptotic cells results in a multitude of 3′-hydroxyl termini of DNA ends. This property can be used to identify apoptotic cells by labeling the DNA breaks with fluorescent-tagged deoxyuridine triphosphate nucleotides (F-dUTP). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3′-hydroxyl ends of double- or single-stranded DNA. A substantial number of these sites are available in apoptotic cells providing the basis for the single step fluorescent labeling and flow cytometric method utilized in the detection of apoptosis. Non-apoptotic cells do not incorporate significant amounts of the F-dUTP owing to the lack of exposed 3′-hydroxyl DNA ends. In consistence with the loss of viability, apoptosis was increased in CRL-2066 and NCI-H23 cells treated by TGZ for 24 h. But apoptosis in CCL-202 cells was not significant, suggesting that unlike in CRL-2066 and NCI-H23 cells, apoptosis is not the major mechanism responsible for the observed growth suppression induced by PPAR-γ activation in CCL-202 cells (Fig. 4).

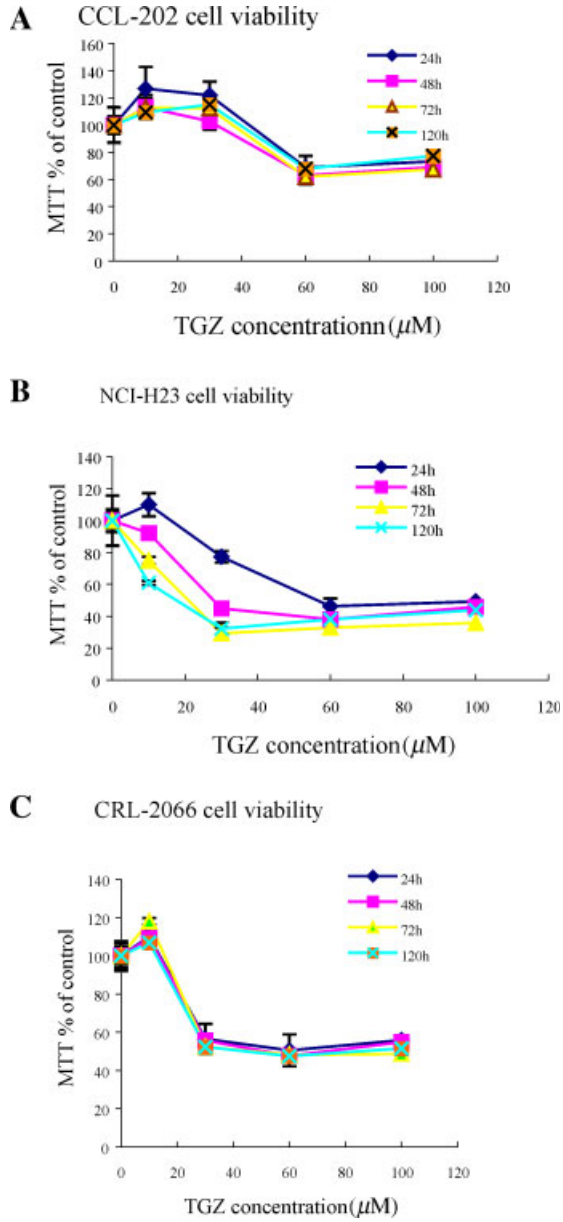


Fig. 2. Effects of PPARγ ligand-TGZ on viability of human lung cells. Human lung cells (CCL-202, NCI-H23, CRL-2066) were treated with TGZ 0, 10, 30, 60, 100 μM for 24, 48, 72, and 120 h, and cell viability was measured by the MTT assay and expressed as % of control culture conditions (0 μM TGZ). The data were represented as mean ± SD for four replicate determinations for each treatment. Experiments were repeated two times with similar results. **A**: CCL-202 cells; (**B**) NCI-H23 cells; (**C**) CRL-2066 cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Ligand-Dependent PPAR-γ Transcriptional Activation by TGZ

The luciferase-based reporter-gene transactivation assay was to assess whether TGZ could

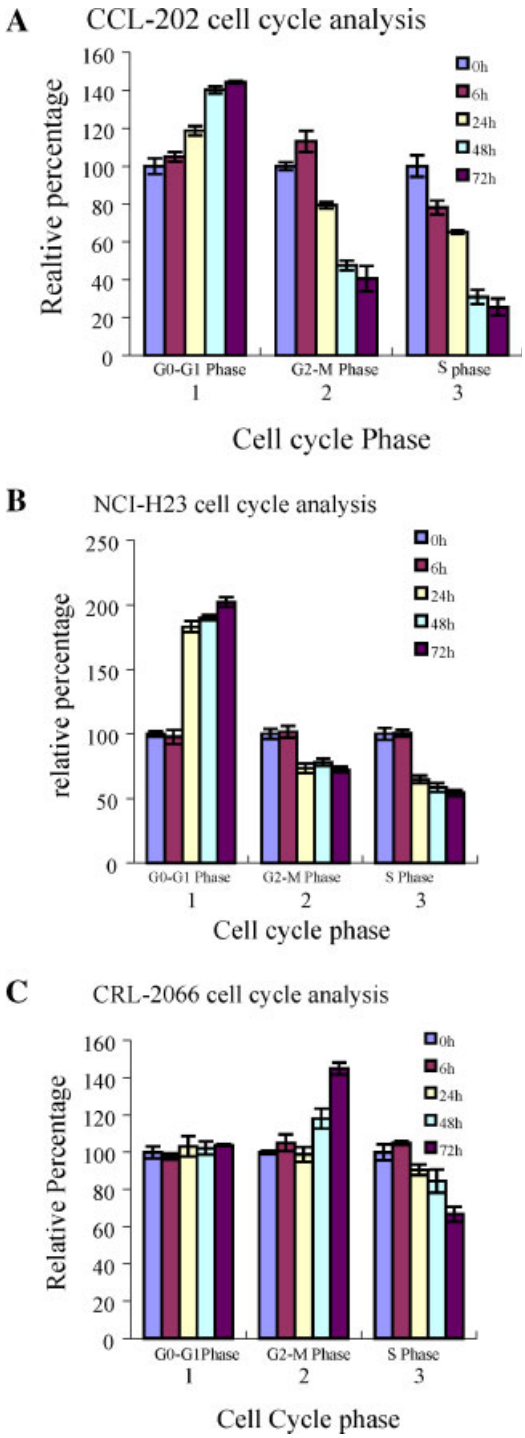


Fig. 3. Effect of TGZ on cell cycle. Cells were treated by 20 μM TGZ for 0, 6, 24, 48, and 72 h. The number of cells in G0/G1, G2/M, and S phases was determined by flow cytometry after staining the cells with PI. The proportion of the each cell phase was determined relative to 0 h cells of the absence of TGZ. Values are mean ± SD of three experiments. **A**: CCL-202 cells; (**B**) NCI-H23 cells; (**C**) CRL-2066 cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

increase the PPAR-γ transcriptional activation. The PPAR-γ response element (PPRE) isolated from the fatty acid transporter protein (FATP) gene was used to drive the expression of the reporter gene. Upon TGZ treatment, the increase of PPAR-γ transcriptional activity was found in three lung cells (Fig. 5).

Effect of TGZ on PPAR-γ Expression in Lung Cell Lines

Real-time PCR (TaqMan-PCR) has increasingly been used as the method of choice for mRNA quantification. It allows the rapid analysis of gene expression from low quantities of starting template. Real-time PCR results showed that TGZ upregulated PPAR-γ mRNA expression in all three lung cell lines, especially in NCI-H23 and CRL-2066 lung cancer cells (Fig. 6). Western blotting assay showed PPAR-γ protein was substantially found in all three-cell lines treated by TGZ (Fig. 6).

Effect of TGZ on the Expression of Bcl-2 Family Members

The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection and quantitation of mRNA species. RPA assay was used to screen possible targets that were altered by TGZ treatment. Among dozens of molecules screened, we found that there was a time-dependent decrease in Bcl-w mRNA expression in CRL-2066 cells treated by TGZ. The decreased expression of Bcl-w mRNA was obviously detected in CCL-202 cells at 2 h time point of TGZ treatment and in NCI-H23 cells at 8 h time point of TGZ treatment. Also, Bcl-2 mRNA expression was downregulated in a time-dependent manner in all three cells studied (Fig. 7).

In consistence with RAP assay, the expression of Bcl-w protein was decreased in a time-dependent manner in CRL-2066 cells treated by TGZ. The expression of Bcl-w protein was not significantly different in CCL-202 cells, but it was markedly reduced at 2 and 72 h time points of TGZ treatment in NCI-H23 cells. For Bcl-2 protein detection, we found a time-dependent downregulation of Bcl-2 expression in CCL-202 cells. And in NCI-H23 and CRL-2066 cells, a decreased level of Bcl-2 protein was also detectable in NCI-H23 and CRL-2066 cells treated by TGZ (Fig. 7).

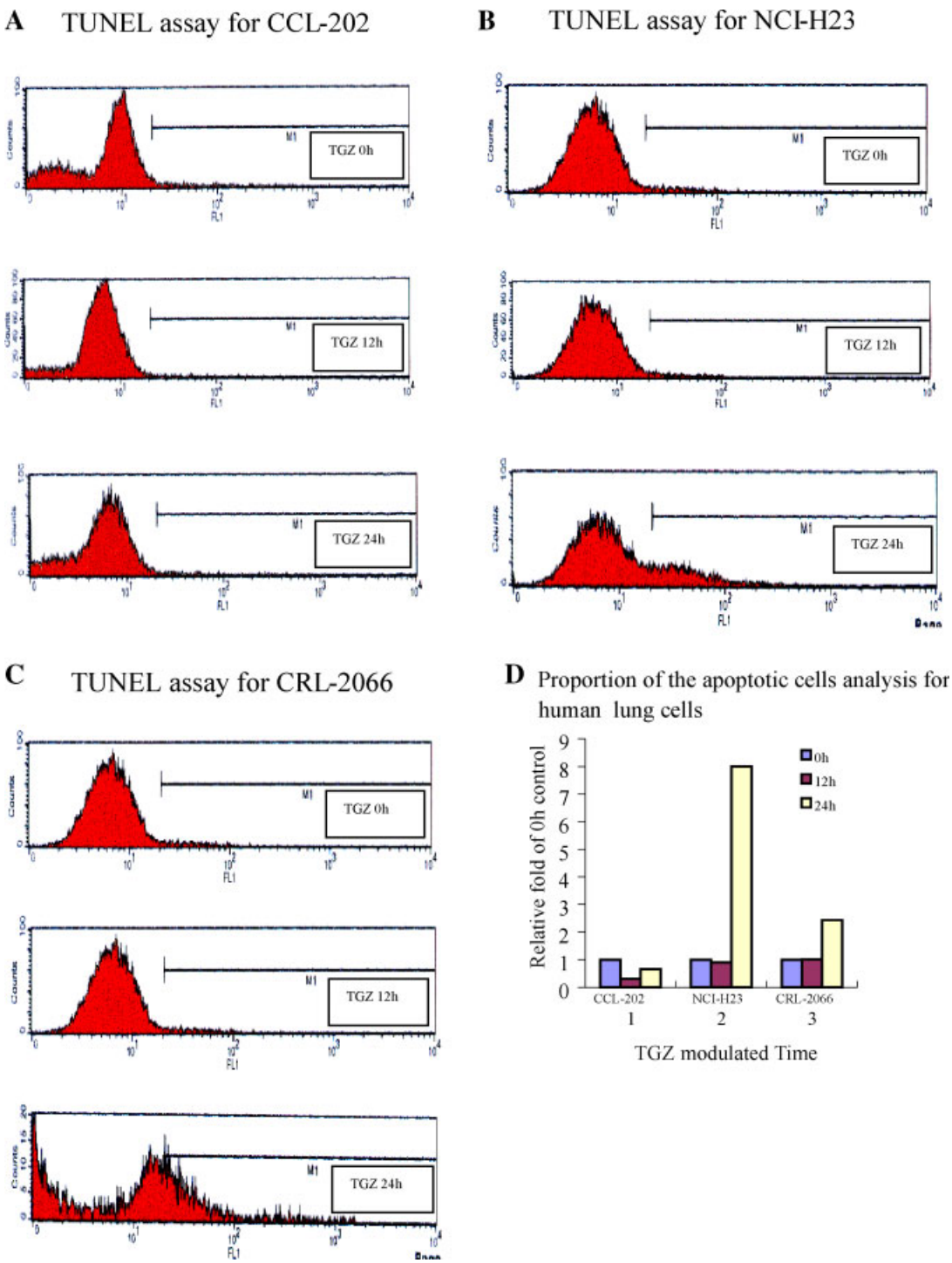
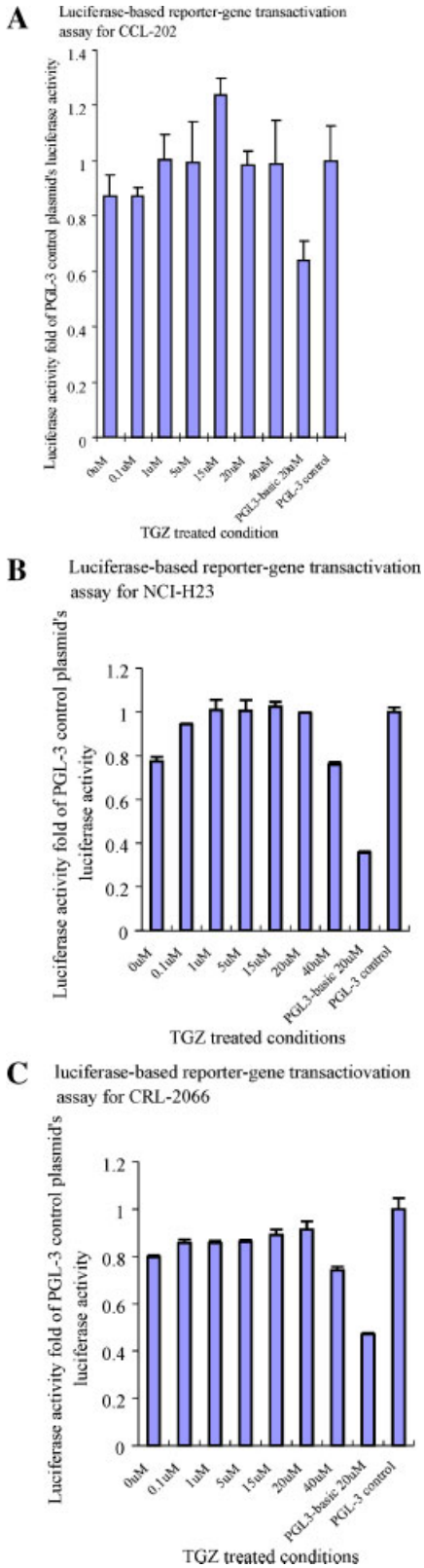


Fig. 4. Effect of TGZ on apoptosis of CCL-202, NCI-H23, and CRL-2066 cells. Cells were treated by 20 μM TGZ for 0, 12, and 24 h. Apoptosis in the three cells was assayed by TUNEL methods using Flow Diagram of APO-DIRECT^{TM} APOPTOSIS ASSAY kit as described under Materials and Methods. **A**: TUNEL assay for CCL-202 cells; (**B**) TUNEL assay for NCI-H23 cells; (**C**) TUNEL assay for CRL-2066 cells. The proportion of the apoptotic cells was determined relative to 0 h cells at the absence of TGZ shown as Figure 4D. Experiments were repeated two times with similar results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



Activation of Mitogen-Activated Protein Kinases (MAPKs) Signal Pathway

It has been know that the MAP kinase family of proteins, known as JNK, ERK, and p38 MAP kinase signals, play an important role in cell survival and apoptosis. The activation of MAPK kinase pathway may exert either anti-apoptotic or pro-apoptotic influence, depending on the cellular context. Western blot was used for analyzing MAPKs in the cells treated with PPAR-γ ligand. The result showed that the time-dependent inhibition of all three-lung cell proliferation by TGZ was associated with sustained ERK1/2 activation. This indicates that sustained ERK1/2 activation may have a growth-inhibitory effect in lung cells. p38 activation was specifically found in CRL2066 treated with TGZ. However, SAPK/JNK activation was decreased by TGZ in the cells tested (Fig. 8).

DISCUSSION

There is an increasing number of publications describing the role of PPAR-γ in human malignancies including thyroid, breast, lung, gastric, colon, and prostate cancers. Although some experiments show a tumor-promoting role of PPAR-γ ligands in certain tumors such as colon cancer, the majority of reports demonstrate an inhibitory effect of PPAR-γ ligands on tumor growth [Chang and Szabo, 2000; Tsubouchi et al., 2000; Wakino et al., 2001; Yang and Frucht, 2001; Yin et al., 2001; Masamune et al., 2002; Keshamouni et al., 2004]. Recently various PPAR-γ ligands have been discovered and studies have shown that their signal transduction pathway may not be the same. Among these PPAR-γ ligands, TGZ or its related compounds has been reported to promote terminal differentiation and morphological changes to well-differentiated and less

Fig. 5. TGZ-induced PPAR-γ activity in lung cells. PPAR-γ activity was analyzed in lung cells after 24 h of TGZ treatment using a luciferase-based reporter-gene transactivation assay. Cells were transiently transfected with either PPRE-tk-Luc or tk-Luc PGL3 basic plasmid, or PGL3 control plasmid as positive control of transfection efficiency and luciferase activity. After 24 h of TGZ treatment under different concentrations, the luciferase activity was assayed using luciferase assay kit as described in Materials and Methods. The data were represented as mean ± SD for three replicate determinations for each group. Experiments were repeated two times with similar results. **A**: CCL-202 cells; (**B**) NCI-H23 cells; (**C**) CRL-2066 cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

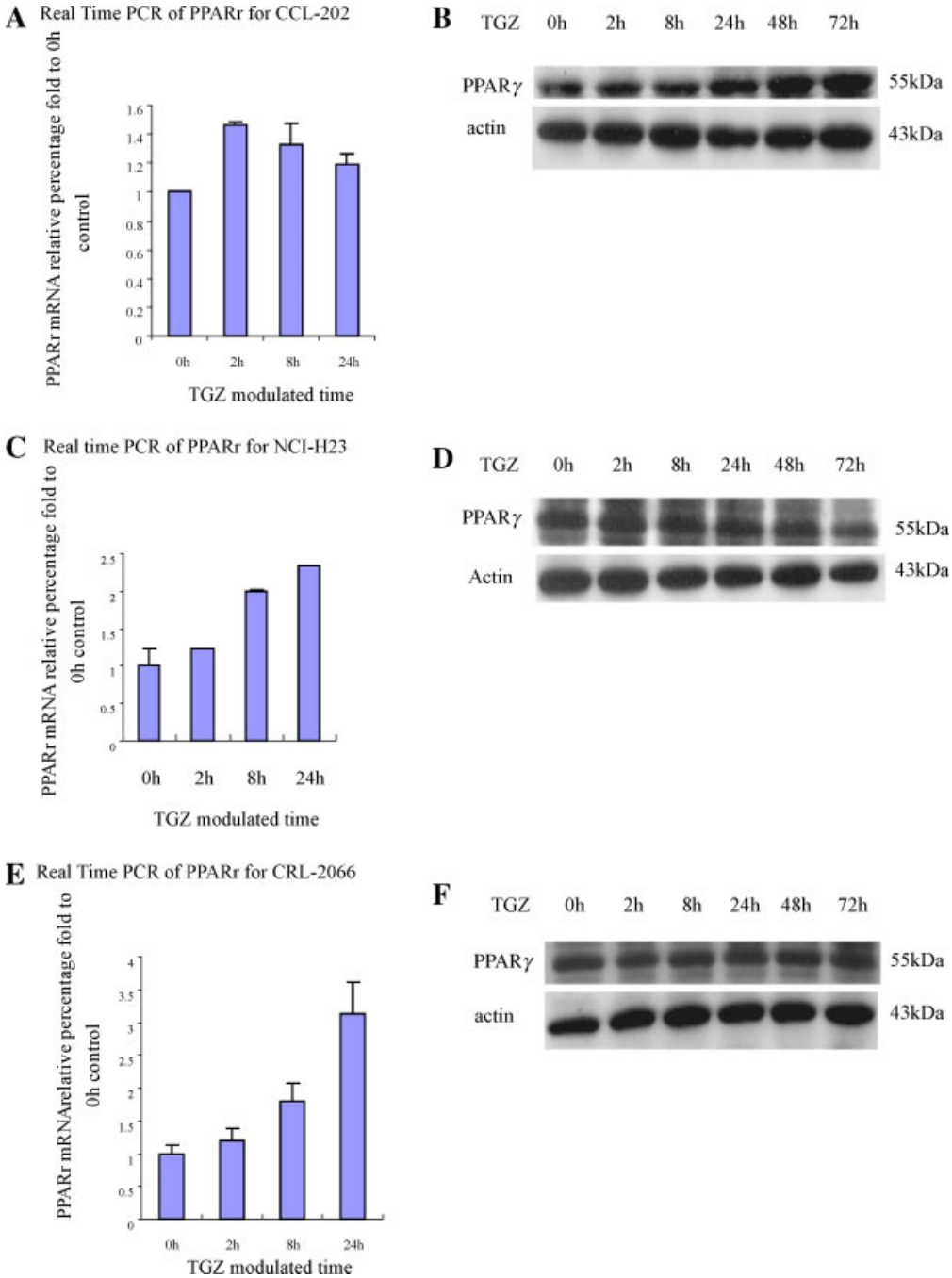


Fig. 6. Effect of TGZ on PPAR-γ expression in lung cell lines. RNA was obtained after cells were treated by 20 μM TGZ for 0, 2, 8, and 24 h. The level of PPARγ expression was determined by real-time PCR. PPAR-γ mRNA was normalized to G3PDH mRNA and presented as fold of 0 h control conditions. The data were represented as mean ± SD for three replicate determinations for each group. Experiments were repeated two times with similar results. Proteins were isolated after cells were treated by 20 μM TGZ for 0, 2, 8, 24, 48, and 72 h. PPARγ protein was determined by Western blotting assay. Equal loading was confirmed by probing with antibodies against actin. Experiments were repeated two times with similar results. **A**, **C**, **E**: Real-time PCR for PPARγ mRNA expression in CCL-202, NCI-H23, and CRL-2066 cells, respectively. **B**, **D**, **F**: Western-blotting assay of PPARγ protein expression in CCL-202, NCI-H23, and CRL-2066 cells, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

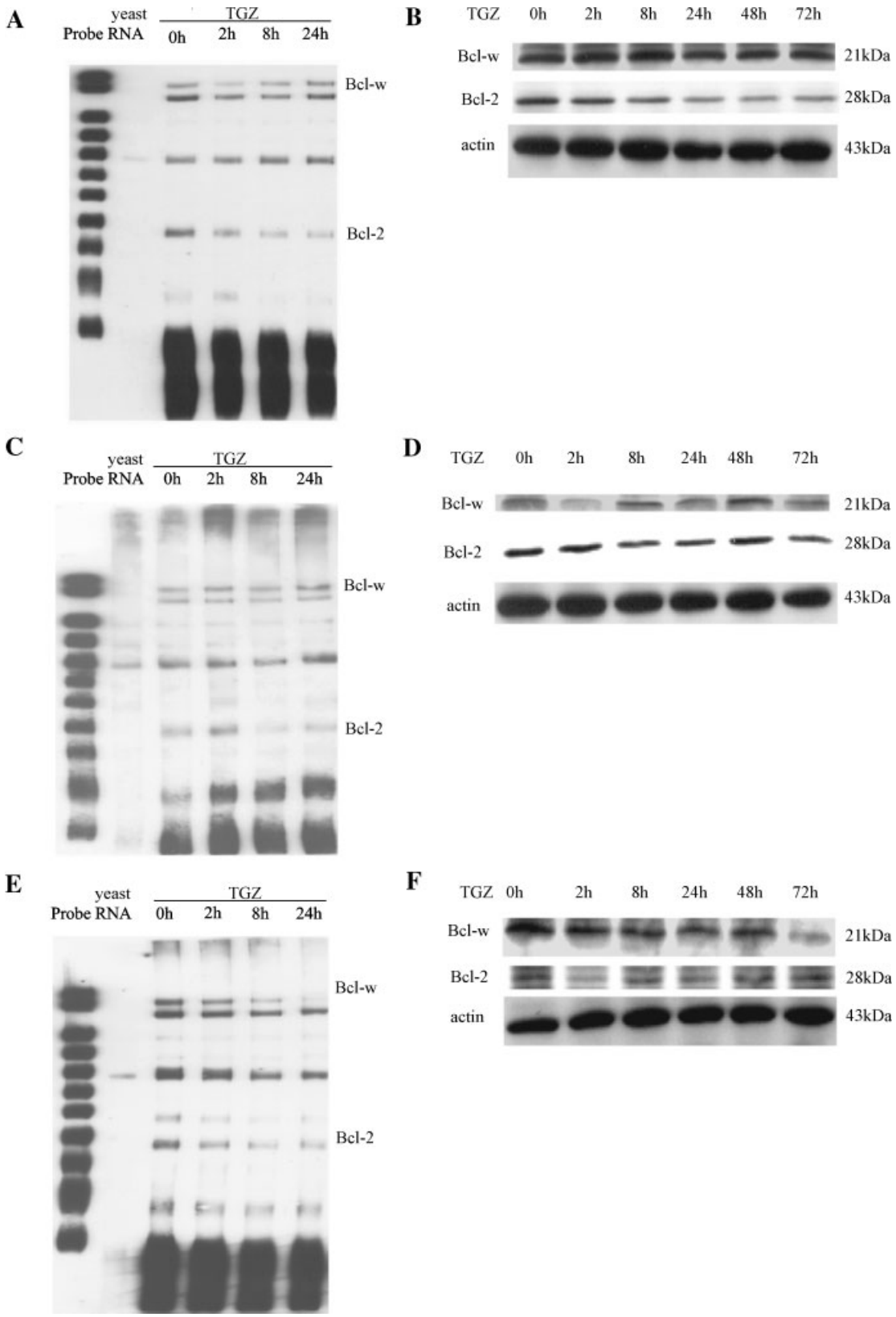


Fig. 7. Effect of TGZ on Bcl-2 family expression in lung cell lines. RNA was obtained after cells were treated by 20 μM TGZ for 0, 2, 8, and 24 h. Bcl-2 family member mRNA was determined by RNase protection (RAP) assay. Proteins were isolated after cells were treated by 20 μM TGZ for 0, 2, 8 h, and 24, 48, and 72 h. Bcl-w and Bcl-2 proteins were determined by Western blotting assay. Equal loading was confirmed by probing with antibodies against actin. **A**, **C**, **E**: RAP assay for CCL-202, NCI-H23, and CRL-2066 cells. **B**, **D**, **F**: Western blotting assay of Bcl-w and Bcl-2 for CCL-202, NCI-H23, and CRL-2066 cells.

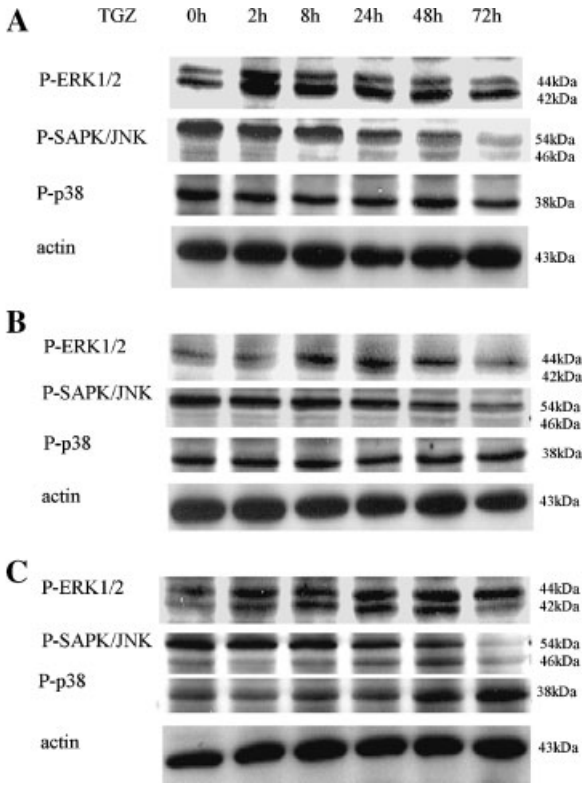


Fig. 8. Effect of TGZ on MAPK kinase activation in CCL-202, NCI-H23, and CRL-2066 cells. Cells were treated by 20 μM TGZ for 0, 2, 8 h and 24, 48, and 72 h. Phospho-ERK1/2 protein, phospho-p38 protein, and phospho-SAPK/JNK protein were determined by Western blotting assay. Equal loading was confirmed by probing with antibodies against actin. **A**: CCL-202 cells; (**B**) NCI-H23 cells; (**C**) CRL-2066 cells.

malignant state. However, the molecular mechanism responsible is not well understood. In the present study, we first confirm that TGZ has an inhibitory role in lung cancer growth via a PPAR-γ-related pathway. We further demonstrate the suppressive effect of TGZ is mainly due to its induction of apoptosis, probably resulting from the alteration of Bcl-2 and Bcl-w expression, which has not been reported before.

Recent studies have demonstrated that apoptosis induced by activation of PPAR-γ was associated with decreased Bcl-2 and NF-κB in human colon cancer and neuroblastoma cell [Chen et al., 2002, 2003; Kim et al., 2003]. Our present experiment in human lung cancer cells reveals that the activation of PPARγ by TGZ is able to downregulate the expression of not only Bcl-2 but also Bcl-w. The biological consequence of decreased Bcl-2 and Bcl-w is the reduction of the anti-apoptotic ability in the cell, as both Bcl-2 and Bcl-w are prosurvival members of the Bcl-2 family [Adams and Cory, 1998; Print et al., 1998]. There is strong evidence that Bcl-2 and Bcl-w interfere with pro-apoptotic BH3-only members of the Bcl-2 family members such as Bax and Bak, thereby preventing the release of proapoptotic mitochondrial proteins [Cheng et al., 2001]. MAPKs are important mediators of intracellular signaling transduction from the plasma membrane into the nucleus. In the present study, we found a sustained activation of ERK1/2 and p38 in lung cancer cells treated with TGZ, especially small lung cell carcinoma cells, CRL-2066. It is documented that PPAR-γ ligands activate ERK in NSCLC cells, murine myoblasts, and gastric mucosal cells [Huang et al., 2002; Slomiany and Slomiany, 2003; Keshamouni et al., 2004] and ERK is involved in the induction of apoptosis [Goillot et al., 1997; Mohr et al., 1998]. For example, ERK activation mediates apoptosis induced by anti-cancer drug Zn^{2+}, peroxinitrate, and ceramide in neuroblastoma SH-SY5Y cells, dopaminergic PC12 cells, and astrocytes [Blazquez et al., 2000; Seo et al., 2001; Kim et al., 2002]. The activation of PPAR-γ coactivator-1, which activates PPAR-γ as well as other nuclear receptors, is positively associated with p38 activity [Puigserver et al., 2001], and interestingly, the overexpression of Bcl-2 can suppress the activation of p38 and subsequently enhance the cell survival [Song et al., 2004]. In our experiment, we have noted that the decreased expression of Bcl-2 and Bcl-w proteins can occur as early as 2 h after TGZ treatment. However, the change of p38 is not observed until 8 h after the treatment in small lung cell carcinoma cells (CRL-2066). Therefore, the reduced level of Bcl-2 and Bcl-w predate the elevated p38, suggesting that p38 may function as a pro-apoptotic molecule located at the downstream of Bcl-2/Bcl-w pathway. Therefore, our findings that PPAR-γ ligand, TGZ, decreases both Bcl-2 and Bcl-w but increases p38 and ERK kinases support the notion that the activation of PPAR-γ reduces the expression of Bcl-2 and Bcl-w to enhance the activity of p38 and subsequently leads to cell growth arrest and apoptosis via a p38 and ERK1/2-related mechanism.

We have also observed that the level of SAPK/JNK was reduced in lung cancer cells treated with TGZ. Like p38 and ERK1/2, SAPK/JNK also plays a role in the cell proliferation and growth. However, SAPK/JNK pathway is negatively regulated by ERK1/2 activity [Shen et al.,

2003]. Although we have not examined the interaction between these two kinases in our present study, the finding that the activation of PPAR-γ exerts opposite effects on ERK1/2 and SAPK/JNK echoes the theory of the negative crosstalk between them. Our finding that after TGZ treatment, lung cancer cells with reduced SAPK/JNK undergo cell growth arrest and apoptosis suggests that SAPK/JNK may have anti-apoptotic role in our experimental model. This assumption is supported by other reports in which SAPK/JNK is known to suppress apoptosis via phosphorylation of the pro-apoptotic Bcl-2 family member proteins [Yu et al., 2004] and its activation is associated with promoting tumoregenesis [Tibbles and Woodgett, 1999; Antonyak et al., 2002].

PPAR-γ was expressed in all the lung cell lines tested at both mRNA and protein levels as evidenced by RT-PCR and Western blotting. In consistence with the loss of viability, apoptosis was increased in small lung cell carcinoma cells (CRL-2066) and non-small lung cell carcinoma cells (NCI-H23) after treatment with TGZ for 24 h. But there was no significant apoptosis found in normal lung epithelial cells (CCL-202), suggesting that unlike in lung cancer cells, CRL-2066, and NCI-H23 cells, apoptosis is not responsible for the observed growth suppression in normal lung cells. The differentiated effects observed between lung cancer cells and normal lung cells may be of therapeutical significance, as TGZ can selectively induce apoptosis in cancer cells without damaging normal cells. The apoptosis found in our study is in contrast to some previous studies using NSCLC cell lines, which reported no significant apoptosis after treatment with either TGZ [Chen et al., 2003] or ciglitazone, another PPAR-γ agonist [Chang and Szabo, 2000]. However, it is in agreement with recent studies in breast and colon and neuroblastoma cancer cells [Elstner et al., 1998; Mueller et al., 1998; Ravi et al., 1998; Sarraf et al., 1998]. The analysis of PPAR-γ activity in these cells suggests that the presence of PPAR-γ protein in these cells is not defective but can be exploited as a potential target for therapy using synthetic PPAR-γ ligand-TGZ. Although without stimulation, the expression of PPAR-γ can occur at both mRNA and protein levels in the cells tested, TGZ can further enhance the level of PPAR-γ in a time-dependent manner. These data again suggest that PPAR-γ is functionally expressed in lung cancer cells. The fact that TGZ can significantly increase the transcriptional activity of PPAR-γ indicates that the inhibition of cell growth and the induction of apoptosis by TGZ is, at least in part, through a mechanism related to PPAR-γ activation.

In summary, we showed that PPAR-γ is expressed in the human lung cells CCL-202, CRL-2066, and NCI-H23 and PPAR-γ ligand TGZ is able to increase its transcription and expression. More importantly, TGZ induces cell growth inhibition through cell cycle growth arrest and induction of apoptosis. Bcl-2 and Bcl-w are involved in the cell growth arrest and apoptosis induced by TGZ. Our study also shows that the increased levels of ERK1/2 and p38 may result from the decreased Bcl-2 and Bcl-w following the activation of PPAR-γ. The reduced level of SAPK/JNK found may also contribute to the apoptosis induced by PPAR-γ ligands through its interaction with Bcl-2 family proteins. Although TGZ may have some effects that are independent of PPAR-γ, the development of apoptosis and its associated molecules altered in the present study are deemed to be PPAR-γ-related since TGZ can activate PPAR-γ transcriptionally in our experimental model. Finally, the differentiated effect of TGZ on lung cancer cells and lung normal cells should make the anti-tumor therapy of TGZ clinically more feasible.

REFERENCES

Adams JM, Cory S. 1998. The Bcl-2 protein family: Arbiters of cell survival. Science 281:1322–1326.

Antonyak MA, Kenyon LC, Godwin AK, James DC, Emlet DR, Okamoto I, Tnani M, Holgado-Madruga M, Moscatello DK, Wong AJ. 2002. Elevated JNK activation contributes to the pathogenesis of human brain tumors. Oncogene 21:5038–5046.

Blazquez C, Galve-Roperh I, Guzman M. 2000. De novo-synthesized ceramide signals apoptosis in astrocytes via extracellular signal-regulated kinase. FASEB J 14: 2315–2322.

Bloch A. 1984. Induced cell differentiation in cancer therapy. Cancer Treat Rep 68:199–205.

Chang TH, Szabo E. 2000. Induction of differentiation and apoptosis by ligands of peroxisome proliferator-activated receptor gamma in non-small cell lung cancer. Cancer Res 60:1129–1138.

Chen GG, Lee JFY, Wang SH, Chan UPF, Ip PC, Lau WY. 2002. Apoptosis induced by activation of peroxisome-proliferator activated receptor-gamma is associated with Bcl-2 and NF-kappaB in human colon cancer. Life Sci 70:2631–2646.

Chen GG, Xu H, Lee JFY, Subramaniam M, Leung KL, Wang SH, Chan UPF, Spelsberg TC. 2003. 15-hydroxy-eicosatetraenoic acid arrests growth of colorectal cancer cells via a

peroxisome proliferator-activated receptor gamma-dependent pathway. Int J Cancer 107:837–843.
Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, Korsmeyer SJ. 2001. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 8:705–711.
Desvergne B, Wahli W. 1999. Peroxisome proliferator-activated receptors: Nuclear control of metabolism. Endocr Rev 20:649–688.
Di-Poi N, Tan NS, Michalik L, Wahli W, Desvergne B. 2002. Antiapoptotic role of PPARbeta in keratinocytes via transcriptional control of the Akt1 signaling pathway. Mol Cell 10:721–733.
Elstner E, Muller C, Koshizuka K, Williamson EA, Park D, Asou H, Shintaku P, Said JW, Heber D, Koeffler HP. 1998. Ligands for peroxisome proliferator-activated receptor-gamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. Proc Natl Acad Sci USA 95:8806–8811.
Feng G, Xu X, Youssef EM, Lotan R. 2001. Diminished expression of S100A2, a putative tumor suppressor, at early stage of human lung carcinogenesis. Cancer Res 61:7999–8004.
Gargiullo P, Wingo PA, Coates RJ, Thompson TD. 2002. MMWR 51:49–53.
Gearing KL, Gottlicher M, Teboul M, Widmark E, Gustafsson JA. 1993. Interaction of the peroxisome-proliferator-activated receptor and retinoid X receptor. Proc Natl Acad Sci USA 90:1440–1444.
Goillot E, Raingeaud J, Ranger A, Tepper RI, Davis RJ, Harlow E, Sanchez I. 1997. Mitogen-activated protein kinase-mediated Fas apoptotic signaling pathway. Proc Natl Acad Sci USA 94:3302–3307.
Gurnell M, Wentworth JM, Agostini M, Adams M, Collingwood TN, Provenzano C, Browne PO, Rajanayagam O, Burris TP, Schwabe JW, Lazar MA, Chatterjee VKK. 2000. A dominant-negative peroxisome proliferator-activated receptor gamma (PPARgamma) mutant is a constitutive repressor and inhibits PPARgamma-mediated adipogenesis. Biol Chem 275:5754–5759.
Han S, Roman J. 2004. Suppression of prostaglandin E2 receptor subtype EP2 by PPARgamma ligands inhibits human lung carcinoma cell growth. Biochem Biophys Res Commun 314:1093–1099.
Huang WC, Chio CC, Chi KH, Wu HM, Lin WW. 2002. Superoxide anion-dependent Raf/MEK/ERK activation by peroxisome proliferator activated receptor gamma agonists 15-deoxy-delta(12,14)-prostaglandin J(2), ciglitazone, and GW1929. Exp Cell Res 277:192–200.
Keshamouni VG, Mattingly RR, Reddy KB. 2002. Mechanism of 17-beta-estradiol-induced Erk1/2 activation in breast cancer cells. A role for HER2 AND PKC-delta. J Biol Chem 277:22558–22565.
Keshamouni VG, Reddy RC, Arenberg DA, Joel B, Thannickal VJ, Kalemkerian GP, Standiford TJ. 2004. Peroxisome proliferator-activated receptor-gamma activation inhibits tumor progression in non-small-cell lung cancer. Oncogene 23:100–108.
Kim SS, Chae HS, Bach JH, Lee MW, Kim KY, Lee WB, Jung YM, Bonventre JV, Suh YH. 2002. P53 mediates ceramide-induced apoptosis in SKN-SH cells. Oncogene 21:2020–2028.
Kim EJ, Park KS, Chung SY, Sheen YY, Moon DC, Song YS, Kim KS, Song S, Yun YP, Lee MK, Oh KW, Yoon DY, Hong JT. 2003. Peroxisome proliferator-activated receptor-gamma activator 15-deoxy-Delta12,14-prostaglandin J2 inhibits neuroblastoma cell growth through induction of apoptosis: Association with extracellular signal-regulated kinase signal pathway. J Pharmacol Exp Ther 307:505–517.
Krueger JS, Keshamouni VG, Atanaskova N, Reddy KB. 2001. Temporal and quantitative regulation of mitogen-activated protein kinase (MAPK) modulates cell motility and invasion. Oncogene 20:4209–4218.
Kubota T, Koshizuka K, Williamson EA, Asou H, Said JW, Holden S, Miyoshi I, Koeffler HP. 1998. Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. Cancer Res 58:3344–3352.
Lambe KG, Tugwood JD. 1996. A human peroxisome-proliferator-activated receptor-gamma is activated by inducers of adipogenesis, including thiazolidinedione drugs. Eur J Biochem 239:1–7.
Mackeigan JP, Collins TS, Ting YPJ. 2000. MEK inhibition enhances paclitaxel-induced tumor apoptosis. J Biol Chem 275:38953–38956.
Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. 1995. The nuclear receptor superfamily: The second decade. Cell 83:835–839.
Masamune A, Satoh K, Sakai Y, Yoshida M, Satoh A, Shimosegawa T. 2002. Ligands of peroxisome proliferator-activated receptor-gamma induce apoptosis in AR42J cells. Pancreas 24:130–138.
Minna JD. 1998. Neoplasms of the lung. In: Fauci AS, Harrison TR, editors. Harrison's principles of internal medicine, 14th edition. New York: McGraw-Hill, Health Professions Division. pp 52–562.
Mohr S, McCormick TS, Lapetina EG. 1998. Macrophages resistant to endogenously generated nitric oxide-mediated apoptosis are hypersensitive to exogenously added nitric oxide donors: Dichotomous apoptotic response independent of caspase 3 and reversal by the mitogen-activated protein kinase kinase (MEK) inhibitor PD 098059. Proc Natl Acad Sci USA 95:5045–5050.
Mueller E, Sarraf P, Tontonoz P, Evans RM, Martin KJ, Zhang M, Fletcher C, Singer S, Spiegelman BM. 1998. Terminal differentiation of human breast cancer through PPAR gamma. Mol Cell 1:465–470.
Parkin DM, Pisani P, Ferlay J. 1993. Estimates of the worldwide incidence of eighteen major cancers in 1985. Int J Cancer 54:594–606.
Pierce GB, Speers WC. 1988. Tumors as caricatures of the process of tissue renewal: Prospects for therapy by directing differentiation. Cancer Res 48:1996–2004.
Print CG, Loveland KL, Gibson L, Meehan T, Stylianou A, Wreford N, de Kretser D, Metcalf D, Köntgen F, Adams JM, Cory S. 1998. Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. Proc Natl Acad Sci USA 95:12424–12431.
Puigserver P, Rhee J, Lin J, Wu Z, Yoon JC, Zhang CY, Krauss S, Mootha VK, Lowell BB, Spiegelman BM. 2001. Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1. Mol Cell 8:971–982.

Ravi RK, Weber E, McMahon M, Williams JR, Baylin S, Mal A, Harter ML, Dillehay LE, Claudio PP, Giordano A, Nelkin BD, Mabry M. 1998. Activated Raf-1 causes growth arrest in human small cell lung cancer cells. J Clin Invest 101:153–159.

Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Singer S, Fletcher C, Spiegelman BM. 1998. Differentiation and reversal of malignant changes in colon cancer through PPARgamma. Nat Med 4:1046–1052.

Sarraf P, Mueller E, Smith WM, Wright HM, Kum JB, Aaltonen LA, de la Chapelle A, Spiegelman BM, Eng C. 1999. Loss-of-function mutations in PPAR gamma associated with human colon cancer. Mol Cell 3:799–804.

Satoh T, Toyoda M, Hoshino H, Monden T, Yamada M, Shimizu H, Miyamoto K, Mori M. 2002. Activation of peroxisome proliferator-activated receptor-gamma stimulates the growth arrest and DNA-damage inducible 153 gene in non-small cell lung carcinoma cells. Oncogene 21:2171–2180.

Seo SR, Chong SA, Lee SI, Sung JY, Ahn YS, Chung KC, Seo JT. 2001. Zn2+-induced ERK activation mediated by reactive oxygen species causes cell death in differentiated PC12 cells. J Neurochem 78:600–610.

Shen YH, Godlewski J, Zhu J, Sathyanarayana P, Leaner V, Birrer MJ, Rana A, Tzivion G. 2003. Cross-talk between JNK/SAPK and ERK/MAPK pathways: Sustained activation of JNK blocks ERK activation by mitogenic factors. J Biol Chem 278:26715–26721.

Shi Y, Hon M, Evans RM. 2002. The peroxisome proliferator-activated receptor delta, an integrator of transcriptional repression and nuclear receptor signaling. Proc Natl Acad Sci USA 99:2613–2618.

Slomiany BL, Slomiany A. 2003. Impedance of *Helicobacter pylori* lipopolysaccharide interference with gastric mucin synthesis by peroxisome proliferator-activated receptor gamma activation involves phosphatidylinositol 3-kinase/ERK pathway. IUBMB Life 55:97–102.

Song YS, Park HJ, Kim SY, Lee SH, Yoo HS, Lee HS, Lee MK, Oh KW, Kang SK, Lee SE, Hong JT. 2004. Protective role of Bcl-2 on beta-amyloid-induced cell death of differentiated PC12 cells: Reduction of NF-kappaB and p38 MAP kinase activation. Neurosci Res 49:69–80.

Spiegelman BM, Flier JS. 1996. Adipogenesis and obesity: Rounding out the big picture. Cell 87:377–389.

Tibbles LA, Woodgett JR. 1999. The stress-activated protein kinase pathways. Cell Mol Life Sci 55:1230–1254.

Tontonoz P, Hu E, Spiegelman BM. 1994. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 79:1147–1156.

Tsubouchi Y, Sano H, Kawahito Y, Mukai S, Yamada R, Kohno M, Inoue K, Hla T, Kondo M. 2000. Inhibition of human lung cancer cell growth by the peroxisome proliferator-activated receptor-gamma agonists through induction of apoptosis. Biochem Biophys Res Commun 270:400–405.

Vamecq J, Latruffe N. 1999. Medical significance of peroxisome proliferator-activated receptors. Lancet 354: 141–148.

Wakino S, Kintscher U, Liu Z, Kim S, Yin F, Ohba M, Kuroki T, Schonthal AH, Hsueh WA, Law RE. 2001. Peroxisome proliferator-activated receptor gamma ligands inhibit mitogenic induction of p21(Cip1) by modulating the protein kinase Cdelta pathway in vascular smooth muscle cells. J Biol Chem 276:47650–47657.

Wick M, Hurteau G, Dessev C, Chan D, Geraci MW, Winn RA, Heasley LE, Nemenoff RA. 2002. Peroxisome proliferator-activated receptor-gamma is a target of nonsteroidal anti-inflammatory drugs mediating cyclooxygenase-independent inhibition of lung cancer cell growth. Mol Pharmacol 62:1207–1214.

Yang WL, Frucht H. 2001. Activation of the PPAR pathway induces apoptosis and COX-2 inhibition in HT-29 human colon cancer cells. Carcinogenesis 22:1379–1383.

Yin F, Wakino S, Liu Z, Kim S, Hsueh WA, Collins AR, Van Herle AJ, Law RE. 2001. Troglitazone inhibits growth of MCF-7 breast carcinoma cells by targeting G1 cell cycle regulators. Biochem Biophys Res Commun 286:916–922.

Yu W, Liao QY, Hantah FM, Sanders BG, Kline K. 2001. Activation of extracellular signal-regulated kinase and c-Jun-NH(2)-terminal kinase but not p38 mitogen-activated protein kinases is required for RRR-alpha-tocopheryl succinate-induced apoptosis of human breast cancer cells. Cancer Res 61:6569–6576.

Yu C, Minemoto Y, Zhang J, Liu J, Tang F, Bui TN, Xiang J, Lin A. 2004. JNK suppresses apoptosis via phosphorylation of the proapoptotic Bcl-2 family protein BAD. Mol Cell 13:329–340.